Plant Pathology (2007) 56, 777–784 Doi: 10.1111/j.1365-3059.2007.01623.x

Detection of lethal yellowing phytoplasma in embryos from coconut palms infected with Cape St Paul wilt disease in Ghana

J. O. Nipah[a], P. Jones[b] and M. J. Dickinson[a]*

[a]Plant Sciences Division, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD; and [b]Plant-Pathogen Interactions Division, Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK

This study investigated the potential of seed transmission of Cape St. Paul wilt disease (CSPWD) in coconuts. PCR amplification was used to assess the distribution of phytoplasmas in parts of West African Tall (WAT) palms infected with CSPWD. Employing phytoplasma universal primer pair P1/P7 in standard PCR, or followed with a nested PCR using CSPWD–specific primer pair G813f/AwkaSR, phytoplasma infection was detected in the trunks, peduncles, spikelets, male and female flowers of four infected WAT coconut palms. Through nested PCR, phytoplasma was also detected in four of 19 embryo DNA samples extracted individually from fruits harvested from three of the four infected palms and was confirmed as CSPWD by cloning and sequencing. Subsequently, CSPWD phytoplasma was again detected in five of 33 embryos from nine infected palms, and in one of eight fruits from two symptomless palms. Fruits from infected palms recorded higher percentage germinations in two field nurseries (average of 71·0%) compared to fruits from healthy palms (average of 57·6%), and matured fruits that had dropped from infected palms showed the same levels of germination as those harvested directly from the palms. This indicates that infected fruits retain the ability to germinate whether harvested or dropped. No phytoplasmas were detected in any of the resulting seedlings and plantlets obtained through embryo *in-vitro* culture. Therefore, although phytoplasma DNA can be detected in embryos, there is as yet no evidence that the pathogen is seed transmitted through to the seedling to cause disease in progeny palms.

Keywords: Cape Saint Paul wilt disease, lethal yellowing, phytoplasma, seed transmission, West African Tall palm

Introduction

Lethal yellowing (LY) is a devastating phytoplasma-associated disease of coconut (*Cocos nucifera*) and at least 35 other palm species in the Americas (McCoy *et al.*, 1983; Harrison *et al.*, 1999). Lethal phytoplasma-associated diseases of coconut have also been reported in East Africa, West Africa and Indonesia (Ashburner *et al.*, 1996; Oropeza & Zizumbo, 1997; Jones, 2002). Because of similarities in symptoms between these diseases and LY, they are collectively referred to as LY-like diseases (Eden-Green, 1997). In West Africa, LY-like diseases have devastated plantations in Nigeria, Cameroon, Togo and Ghana (Dery *et al.*, 1997; Mpunami *et al.*, 1999). All these lethal diseases of coconut have been associated with phytoplasmas.

In Ghana, the coconut palm is the most important crop along the coastal belt, and offers employment opportunities to several disadvantaged groups, including women and landless poor in the rural coastal communities. It is the main source of livelihood, providing food, fuel-wood, drink, edible oil, fibre, animal feed and building materials with minimum capital outlay (Ofori & Nkansah-Poku, 1997). The occurrence of an LY-like disease which is locally referred to in Ghana as the Cape Saint Paul wilt disease (CSPWD) has resulted in severe economic hardships in the coconut producing communities along the coastal belts in affected West African countries. CSPWD, like other LY-like diseases, is characterized by premature nut fall and necrosis of both newly opened and unopened inflorescences, followed by successive yellowing of the crown, progressing from the lower leaves until the whole crown turns brown and breaks off leaving the bare trunk (McCoy *et al.*, 1983). The local West African Tall (WAT) variety, which is the major type in almost all plantations in Ghana, is highly susceptible to the disease, with 100% devastation recorded in many plantations. Against this background the government of Ghana, in collaboration with the governments of France and Côte d'Ivoire, launched an intensive research programme in 1981, which led to the identification of some individuals and ecotypes with high

*E-mail: matthew.dickinson@nottingham.ac.uk

Accepted 3 January 2007

resistance/less susceptibility to the disease in Ghana. The Sri Lanka Green Dwarf (SGD) variety was ranked as highly resistant and the Vanuatu Tall (VTT) variety also exhibited less susceptibility to CSPWD. The hybrid between the Malayan Yellow Dwarf (MYD) and VTT was also less susceptible (Dery *et al.*, 1997, 2005; Nipah, 2000).

Although coconut breeding programmes are constantly searching for disease resistant germplasm, variations in phytoplasmas associated with LY-like diseases may contribute to differential resistance among coconut ecotypes in different parts of the world (Ashburner & Been, 1997; Harrison & Oropeza, 1997). The identification of promising genotypes in Ghana could hold major prospects for coconut producing countries in the West Africa sub region, which are likely to be affected by the same strain of phytoplasma. However, there is a major concern that LY-like phytoplasmas, including CSPWD, could be seed transmitted. This possibility has wide ranging implications for the coconut breeding programme in Ghana, because almost all varieties introduced into the country for breeding purposes have been planted within areas which are prone to disease attack. Therefore, if CSPWD is seed transmissible, progenies of any breeding programmes could not be planted in disease-free regions, since it would amount to introducing the phytoplasma into these areas. Such fears have led to research programmes involving the production of pure seeds from varieties located originally in CSPWD areas, for conservation in disease-free regions within the country, being turned down by funding authorities. The local coconut research programme is also prevented from exporting even resistant germplasm to neighbouring countries for collaborative research purposes, because officials of the Plant Protection and Regulatory Services do not have the necessary scientific basis to make such important decisions concerning safe movement of germplasm.

By definition, phytoplasmas are phloem-restricted mollicutes. Dollet (2002) indicated that of more than 600 phytoplasma diseases known at that time, none had been reported as being transmissible by seed, and that the same applied for phloem restricted viruses. However, Harrison & Oropeza (1997) reported that pathogen-specific PCR revealed the presence of lethal yellowing phytoplasma in at least one seed from fruits of three coconut palms exhibiting primary stage symptoms, following 40 cycles of PCR.

Such findings, however, have not resolved the controversy of possible seed transmission. The Food and Agricultural Organization (FAO) and the International Board for Plant Genetic Resources (IBPGR) recommends that exchanges of coconut germplasm be carried out in the form of zygotic embryo *in vitro* cultures, in order to remove the risk of introducing new pathogens via the nut (Anon., 2002). However, whilst embryo culture may reduce such risks, there is no scientific basis to conclude that it will completely remove the possibility of seed transmission, and not all coconut producing countries have the facilities or the expertise to effectively handle embryo cultured germplasm. Furthermore, the recommendation appears to be based on the belief that infected embryos are unlikely to germinate. This study was therefore carried out with the broad objective of determining whether CSPWD phytoplasma can be seed transmitted. This involved an initial assessment of the extent of distribution of CSPWD phytoplasma DNA in parts of the inflorescence of an infected palm, with particular interest in the female flowers which eventually develop into the fruit, followed by an assessment of the presence of the pathogen in the embryos from infected palms. The chances of such embryos germinating and the possibility of the resulting seedlings containing phytoplasma were also studied.

Materials and methods

Plant DNA Extraction

Genomic DNA was extracted from the trunks and inflorescences of healthy and CSPWD-infected WAT coconut palms in Ghana using a CTAB extraction method. Trunk samples were obtained with the aid of a motorised hand-held drill, which chips out phloem tissues in the form of sawdust. To avoid cross contamination from plant to plant, the drill bit was rinsed in 0·5% sodium hypochlorite solution, flamed and cooled in alcohol between palms. Trunk tissue (1 g) was mixed with 5 mL of CTAB extraction buffer (20 mM EDTA pH 8·0, 100 mM Tris-HCl pH 8·0, 2% CTAB, 1·4 M NaCl, 2-mercaptoethanol in sterile distilled water). To obtain DNA from the peduncle, spikelets, male flowers and female flowers of each inflorescence, and from the apical leaves of field nursery seedlings, 1 g tissue from each of the components was crushed with a hammer in 5 mL of CTAB buffer while sealed in transparent polythene bags. Samples were then left in the CTAB for a period of 48 h, with occasional mixing by inversion. One millilitre of each sample was poured into a 2 mL tube and DNA was extracted with chloroform/iso-amyl alcohol and precipitated with ethanol using an extraction protocol of Daire *et al.* (1997). Total DNA from whole embryos, leaves and roots of embryo cultures was extracted using the DNeasy Plant Mini Kit (Qiagen) protocol. Embryos were excised with the aid of sterile cork borers.

PCR amplification of phytoplasma DNA

Total plant DNA samples were screened for the presence of phytoplasma by PCR in 25 μL reactions using 'Ready To Go PCR beads'™ (Amersham Pharmacia Biotech) containing 15 ng template DNA and 100 ng of each primer in an MJ Research PTC200 thermocycler. Forward primer P1 and reverse primer P7, which are general for phytoplasmas (Deng & Hiruki, 1991; Smart *et al.*, 1996) were used to amplify 1·8 kb of the 16S rRNA phytoplasma gene. Amplification was carried out with an initial denaturing temperature of 94°C for 1 min 30 sec, followed by 35 cycles of 94°C for 30 sec, 56°C for 50 sec and 72°C for 1 min 30 sec, and a final extension at 72°C for 10 min. Where no phytoplasma-specific band was amplified after the standard PCR with the general primers (as was the case in all embryo samples) a nested PCR was carried out

with CSPWD phytoplasma-specific primer Ghana 813f (5′-CTA AGT GTC GGG GGT TTC C-3′) and Awka SR (5′-TTG AAT AAG AGG AAT GTG G-3′) (Tymon, 1995). Except for an annealing temperature of 53°C in the nested reaction, all other PCR conditions were as before. PCR products were visualized on 1% agarose gels stained with ethidium bromide.

Restriction Fragment Length Polymorphism (RFLP), cloning and sequencing

Samples yielding visible DNA bands after the nested PCR were examined by RFLP using restriction enzyme *Rsa*I. Five microlitres of PCR product was digested for 4 h at 37°C in a 10 μL reaction volume using 0·5 units of enzyme. Digested products were run on 2% agarose gels stained with ethidium bromide. Nested PCR products were cloned using the Promega pGEM®-Teasy Vector System cloning protocol (Promega). Colonies were amplified by PCR with the universal primers M13For and M13Rev included in the kit. The cloned products were cleaned using Gen Elute™ PCR Clean-Up Kit (Sigma) before sequencing. Sequences were processed on both strands using a Beckman Quickstart kit technology which uses WellRed Dye chemistry (infra-red dyes), with a CEQ 8000 Genetic Analysis System, manufactured by Beckman Coulter.

Germination of fruits in field nurseries and embryo culture

Matured seeds which were harvested directly from CSPWD infected palms and those that had already dropped from the palms were germinated either conventionally on seedbeds or through embryo *in-vitro* culture. Two seedbed nurseries were established in disease-free conditions within sealed mesh cages following recommendations of the STANTECH manual (Santos *et al.*, 1994). The cages and nursery beds were regularly sprayed with insecticides at 14-day intervals to prevent contact with insects. The rates of germination were noted and leaves were sampled from germinated seeds three and six months after nursing for PCR analysis. The upper parts of apical leaves were sectioned as close as possible to the growing point without killing the seedling, and crushed in CTAB buffer for DNA extraction. For embryo culturing, endosperm cylinders with embryos were sterilised for 20 minutes in 10% sodium hypochlorite solution and washed in three changes of sterile water before the embryos were removed under sterile conditions. Embryos were surface-sterilized in 1% sodium hypochlorite for 10 minutes, rinsed three times and inoculated on liquid Y3 media as developed by the PCA (Philippine Coconut Authority) and the CPCRI (Central Plantation Crops Research Institute) which had been slightly modified by excluding activated charcoal and adding 1 mg L^{-1} BAP (6-benzylaminopurine) and 0·5 mg L^{-1} NAA (Batugal & Engelmann, 1998). Cultures were incubated in darkness for four weeks after which developing embryos were transferred to fresh PCA Y3 liquid medium and maintained in a 16 h photoperiod at 22°C. The plantlets were transferred to fresh medium after every 4–6 weeks, and those with differentiated leaves were sampled. A total of two plantlets from healthy palms, three from symptomless palms and 12 from diseased palms were sampled 3 and 6 months after culturing and tested for the presence of phytoplasma. DNA was extracted from sections of leaves, leaf sheaths and roots.

Results

Detection of phytoplasma in different parts of infected coconut palms

To assess the distribution of phytoplasma in parts of the inflorescence of an infected palm, total DNA extracted from the trunks and inflorescences of one healthy palm and four CSPWD-infected coconut palms (palms 1–4) were screened by PCR. The healthy palm was located at Ayinasie, approx. 10 km from the disease front. A total of nineteen matured embryos were harvested from palms 1, 2 and 3, and one from each of these three diseased palms was included in the PCR screening. Palm 4 did not have any matured fruits. After a standard PCR with general primers P1 and P7, bands of 1·8 kb, the expected size for the partial ribosomal operon of the phytoplasma, were clearly amplified in total DNA extracted from trunks, peduncles, spikelets, male flowers and female flowers of infected palms (Fig. 1). In the case of female flowers, only one of three samples from infected palms showed the band. No visible band was present in any of the three embryos included in the screening and in the organs of the healthy control.

Negative P1/P7 PCR reactions were used as templates in a nested PCR, using primers G813F and Awka-SR. Visible bands matching the size of the 16S rRNA gene segment of the CSPWD (889 bp) were observed in all floral samples from infected palms. The embryo from palm 3, as well as male and female flowers from the healthy palm, also showed visible bands of the same size (Fig. 2).

Assessment of 16 embryos from diseased palms for the presence of phytoplasma

Following the observation of bands in products from all parts of the inflorescence, and in one of the three embryo products in the initial study, a more comprehensive screening was carried out on the remaining 16 embryos harvested from palms 1, 2 and 3. These comprised three embryos from palm 1, 11 from palm 2 and two from palm 3. DNA from the peduncles of the three palms, a positive control and a negative control were included as checks. After PCR using primers P1 and P7, products from all three peduncles and the positive check produced discrete bands of 1·8 kb. No bands were visible in any of the 16 embryos. When all the products were subjected to nested PCR using primers G813f and AwkaSR, three of the 16 embryos produced the right sized bands (889 bp). These included one of the three embryos from palm 1 and two of the 11 embryos from palm 2 (Fig. 3).

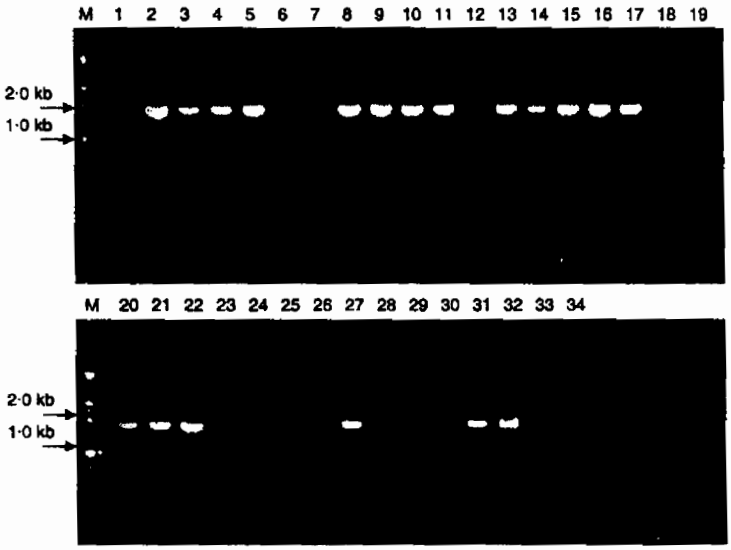

Figure 1 PCR amplification from coconut trunks, parts of the inflorescence and embryos with primers P1 and P7. The presence of a 1·8 kb band indicates the presence of phytoplasma DNA in the sample. DNA samples from: lane 1, healthy palm trunk; 2–5, trunks of palms 1–4; 6, healthy palm peduncle; 7–11 peduncles of palms 1, 2, 3 (a and b), 4; 12, healthy palm spikelet; 13–17, spikelets of palms 1, 2, 3 (a and b), 4; 18, healthy palm male flowers; 19–23, male flowers of palms 1, 2, 3 (a and b), 4; 24, healthy palm female flowers; 25–27, female flowers of palms 1, 3 and 4; 28–30, embryos of palms 1–3; 31 and 32, positive control; 33 and 34, negative control. M = 1 kb ladder. Infected palms 1 & 2 were at the first stage of the disease while 3 & 4 were at the second stage.

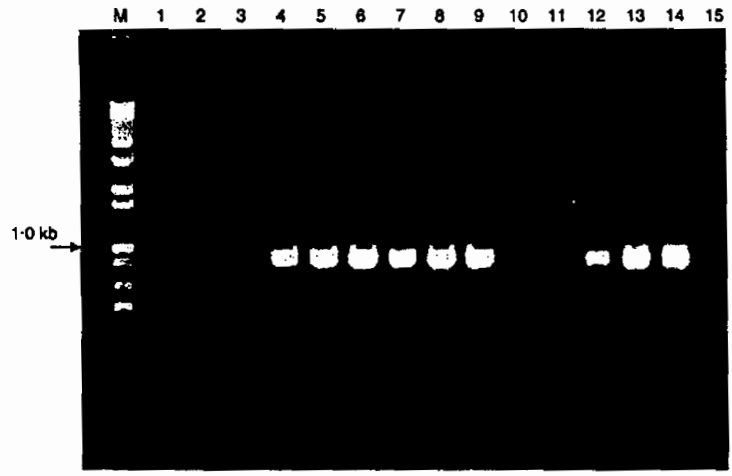

Figure 2 Nested PCR amplification using primers Ghana 813f and Awka-SR of samples that did not show any band in Figure 1. A 889 bp band indicates the presence of phytoplasma DNA. DNA samples from: lane 1, healthy trunk; 2, healthy peduncle; 3, healthy spikelet; 4, healthy male flower; 5, healthy female flower; 6, palm 1 peduncle; 7, palm 4 male flower; 8, palm 1 female flower; 9, palm 3 female flower; 10, palm 1 embryo; 11, palm 2 embryo; 12, palm 3 embryo; 13 and 14, positive control; 15, negative control.

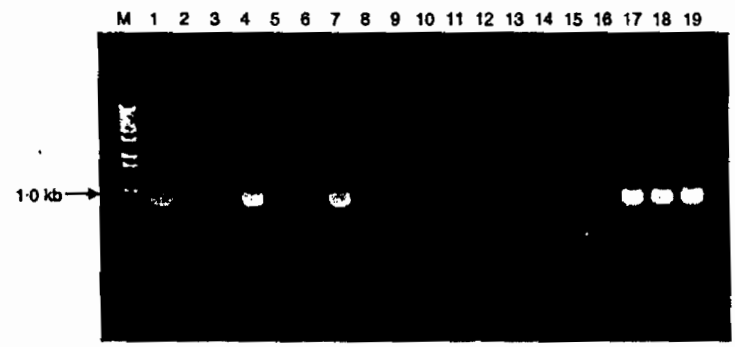

Figure 3 Nested PCR products from embryos and peduncles using primers Ghana 813F and Awka-SR. DNA samples from: lanes 1–3, palm 1 embryos 1–3; 4–14, palm 2 embryos 1–11; 15–16 palm 3 embryos 1 and 2; 17–19 peduncles from palms 1, 2 and 3.

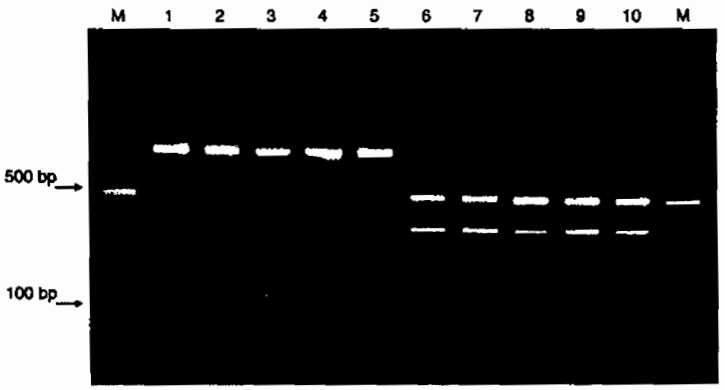

Figure 4 Restriction digestion of nested PCR products obtained from embryo and coconut palm trunk samples. Lanes 1–5, uncut nested PCR products amplified with Ghana813f/AwkaSR; lanes 6–10, fragments resulting from digestion with *Rsa*I. Lanes 1, 2, 6 and 7 were from embryo samples and lanes 3, 4, 5, 8, 9 and 10 were from trunk samples.

Table 1 Seed germination of coconut palm in field nurseries

	Healthy		Diseased		Symptomless	
	Nursed embryos	Germination (%)	Nursed embryos	Germination (%)	Nursed embryos	Germination (%)
Sekondi Nursery						
Dropped	2	1 (50·0)	11	9 (81·8)	–	–(–)
Harvested	13	8 (61·5)	12	10 (83·3)	6	2 (33·3)
Total	15	9 (60·0)	23	19 (82·6)	6	2 (33·3)
Accra-BNRI Nursery						
Dropped	4	2 (50·0)	21	14 (66·7)	–	–(–)
Harvested	14	8 (57·0)	17	11 (64·7)	6	3 (50·0)
Total	18	10 (55·6)	38	25 (65·8)	6	3 (50·0)
Overall Totals	33	19 (57·6)[a]	61	44 (72·1)[a]	12	5 (41·7)

[a]Means differ significantly ($P < 0·05$) according to Fishers LSD test.

RFLP, cloning and sequencing

To confirm that the observed bands resulting from the nested PCR were of phytoplasma origin, they were digested with the restriction enzyme *Rsa*I. The enzyme should produce two resolvable fragments of 498 bp and 326 bp and four smaller ones of 4, 16, 21 and 24 bp, which cannot be resolved on the 2% agarose gel. *Rsa*I digestion of the nested PCR products resulted in the two expected fragments (Fig. 4).

Cloning and sequencing of these PCR products also showed all the nested products to be of the same strain. Blast results from the sequences of products from embryos and trunks showed perfect alignment with the 16S rRNA gene of the CSPWD phytoplasma (Acc. No. Y13912), which is also designated as LDG-strain (lethal disease of Ghana).

Germination of seed from infected palms, embryo culture and seedling assessment

Following detection of phytoplasmas in some embryos from the three palms, fresh collections of seeds were made from two healthy, two symptomless and nine infected palms. Healthy palms were located at Takinta, approx. 100 km from the disease front, and symptomless palms were located in high disease focal areas but looked apparently healthy. DNA was extracted from the embryos of randomly selected seeds for nested PCR analysis to confirm the presence of phytoplasma ahead of the germination tests. Out of a total of five embryo DNA samples from the healthy palms, eight from symptomless palms and 33 from diseased palms, one positive was recorded from the symptomless palms and five from diseased palms (Fig. 5).

The remaining seeds were classified and treated as shown in Table 1. A total of 33 seeds from the healthy palms, 12 from symptomless palms and 61 from diseased palms, were nursed in mesh cages at two disease-free locations; Sekondi and Biotechnology and Nuclear Research Institute (BNRI-Accra). While 19 of the 33 healthy seeds (57·6%) germinated, significantly more, 44 of the 61 seeds (72·1%) germinated from diseased palms. Germination of five seeds was also recorded among the 12 seeds from symptomless palms (41·7%). In both the Sekondi and BNRI-Accra nurseries, higher percentage germination was recorded among the seeds from diseased palms compared to those from healthy palms (Table 1). Both harvested and dropped seeds from diseased palms showed approximately equal chances of germination. However, dropped seeds from the healthy palm were too few to compare with harvested ones.

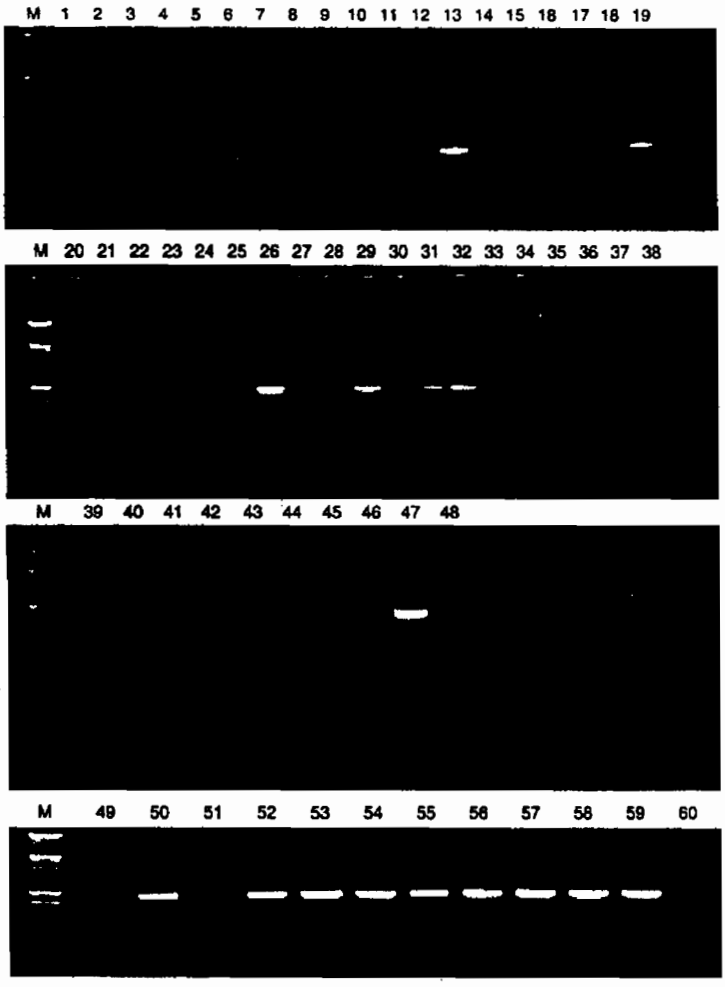

Figure 5 Nested PCR products from embryos and corresponding coconut palm trunks. Phytoplasma DNA was amplified with primers P1/P7 followed with G813F/Awka-SR. Lanes 1–5, embryo samples from two healthy palms; 6–13, embryo samples from two symptomless palms; 14–46, embryo samples from nine diseased palms; 47, positive control; 48, negative control; 49, healthy trunk; 50, symptomless palm 1 trunk; 51, symptomless palm 2 trunk; 52–60, diseased palms 1–9 trunks.

Nested PCR performed on all germinated seedlings from both Sekondi and BNRI-Accra as well as plantlets from embryo cultures after 3 and 6 months of nursing and culturing, respectively, failed to detect the presence of phytoplasma in any of the samples.

Discussion

Plant-to-plant transmission of phytoplasmas occurs primarily during feeding activity by inoculative vector insects, and in some cases by vegetative propagation of infected plant materials or by graft inoculation (Kirkpatrick, 1992). However, Botti & Bertaccini (2006) have recently reported the seed transmission of phytoplasmas into 2- to 3-week-old seedlings of tomatoes and winter oilseed rape, and 3- to 5-month-old plantlets of lime. Seed transmission has generally been accepted as highly unlikely mainly because phloem sieve elements of plants in which phytoplasma reside do not have direct connection to embryos. Furthermore since coconut LY-like diseases usually include premature nut fall, the production of viable seeds may not be possible once disease infection has occurred. However, this view is not completely true. Harrison *et al.* (1994) detected phytoplasmas in some parts of the coconut inflorescence, and in this study reported here pathogen DNA has been detected in all parts of the coconut inflorescence, including the female flowers. Since it is the same cells or tissues of the female flowers that divide into specialized cells in the fruit, in theory it should be possible for phytoplasma DNA to be identified in any tissue of a fruit which develops from an infected female flower.

Field studies have established that there is an incubation period between phytoplasma infection of a palm and the onset of a disease. This period could be very variable across palms, varieties and strains of phytoplasma causing the LY-like diseases and may be dependant on the age of the palm. In the highly susceptible Jamaica Tall variety, incubation periods of 112–262 days have been reported (Dabek, 1975). In one study in Ghana VTT palms that

initially tested positive for phytoplasmas in trunk tissues have not yet developed symptoms after six years of that study, and periods of up to 844 days have been recorded between the first detection of phytoplasma and the onset of disease in MYD × VTT hybrids (OPRI, 1998; Nipah, 2000), although such reports of apparent tolerance have not as yet been confirmed by others. During this period where development in the plant remains normal, tissues of the inflorescence, including female flowers and therefore developing fruits, could be infected with phytoplasma. Indeed in this study, a trunk-sample and one from an embryo from an apparently symptomless plant tested positive, although it has not been confirmed whether this palm eventually succumbed to disease, or was due to a sampling error. Furthermore not all fruits drop prematurely at the onset of disease symptoms. In this study, viable seeds were harvested from infected palms some of which were at the third stage of the disease. Again surveys of CSPWD in Ghana have suggested the possibility of remission of disease symptoms, where in a particular case, palms with apparent CSPWD symptoms completely recovered from earlier symptoms after four months of disease incidence, although these palms were not tested at any stage with PCR-based methods to confirm the presence of phytoplasma DNA (Dery *et al.*, 1999; Nkansah-Poku *et al.*, 2003). Finally, palms infected with LY-like diseases could remain at the early stages of the disease for extended periods before they eventually succumb and die. Under such circumstances, fruits that do not drop prematurely could continue their normal development.

The presence of phytoplasma in the embryos of coconut palms has already been demonstrated or reported by other researchers (Harrison & Oropeza, 1997; Cordova *et al.*, 2003). This study has clearly confirmed the situation. The presence of visible bands of the correct size in two samples from a supposed healthy palm initially made it uncertain as to whether the bands, including the one observed in the embryo, were really the expected amplified segment of the 16S rRNA gene of the phytoplasma. Subsequent RFLP analysis and sequencing of the nucleotide segment confirmed the bands as being of the CSPWD phytoplasma sequence only. It is worth noting that healthy samples in the initial studies were collected from plantations in Ayinasie, located only about 10 km from the disease front. Healthy samples from Takinta, which is farther away from the disease front, did not show any phytoplasma-specific band. In this study, one of three embryos harvested from palm 3 tested positive for phytoplasma DNA in the initial studies. In the subsequent study involving 16 embryos, two of the 11 embryos from palm 2 and one of the three embryos from palm 1 tested positive for phytoplasma DNA. Therefore, at least one embryo from each palm showed the presence of phytoplasma, even though only a small number of seeds were included from palms 1 and 3. Similarly, in the work done by Harrison & Oropeza (1997), at least one phytoplasma positive embryo was detected among seeds from each of three infected palms. The final study involving 33 embryos again recorded five positives and also one positive was recorded from the eight symptomless samples. Considering such consistency in the records of phytoplasma DNA detected in the coconut embryo, there can no longer be any uncertainty about the ability of phytoplasma DNA to find its way into embryos. However, the mechanism for this remains unclear, and until there is microscopic evidence to confirm the presence of the organism there remains a possibility that it is the phytoplasma DNA that enters the embryos and is somehow protected against nuclease digestion.

The questions that now need to be addressed are whether such infected embryos can germinate, and also whether the phytoplasma DNA found in the embryo is associated with viable organisms that can survive the process of seedling development and so result in disease. Some valid inferences could be made from the germination percentages recorded in the two field nurseries. Two main seed sampling tests were performed in this study. In the first set of seeds from infected palms, four out of 19 (21%) tested positive for phytoplasma. In the second set, five out of 33 (15%) tested positive. If the assumption is made that infected embryos cannot germinate then one would expect seeds from infected palms to generally record lower germination percentages in comparison to those from a healthy source. However, the opposite was the case in this study, and supports other germination records (data not shown). Furthermore the ability to germinate was found to be independent of whether the matured seed had dropped or was freshly harvested from the diseased palm. This indicates that matured embryos infected with phytoplasma can still retain the ability to germinate regardless of whether the seed had already dropped from the stalk or not. This concurs with the previous findings of Romney (1983), who showed that when 600 seedlings raised from seed nuts collected from diseased Jamaican Tall palms were planted in disease-free areas, they remained disease-free, although no diagnostic tests were performed to detect phytoplasmas. Since the current study failed to detect any evidence of the presence of phytoplasma in any of the seedlings and plantlets, it is concluded that although phytoplasma DNA can be detected in the embryo, there is as yet no evidence that the pathogen can be seed transmitted through the seedling to cause disease in the resultant palm.

References

Anon., 2002. *Proceedings of the Expert Consultation on Sustainable Coconut Production through Control of Lethal Yellowing Disease, Kingston, Jamaica 2002*. CFC Technical Paper No 18.

Ashburner GR, Been BO, 1997. Characterization of resistance to lethal yellowing in *Cocos nucifera* and implications for genetic improvement of this species in the Caribbean region. In: Eden-Green SJ, Ofori F, eds. *Proceedings of an International Workshop on Lethal Yellowing-Like Diseases of Coconu, Elmina, Ghana, 1995*. Chatham, UK: Natural Resources Institute, 173–83.

Ashburner GR, Córdova II, Oropeza CM, Illingworth R, Harrison NA, 1996. First report of coconut lethal yellowing disease in Honduras. *Plant Disease* 80, 960.

Batugal PA, Engelmann F, 1998. Coconut embryo *in vitro* culture. *Proceedings from the Workshop on Embryo Culture,Banao, Philippines 1997*. Serdang, Malaysia: IPGRI-APO.

Botti S, Bertaccini A, 2006. Phytoplasma infection through seed transmission: Further observations. In: Ayling RD, Citti C, Nicholas RAJ, eds. *Proceedings of the 16th International Congress of the International Organization for Mycoplasmasmology, 2006*. Cambridge, UK: Veterinary Laboratories Agency, 76.

Cordova I, Jones P, Harrison NA, Oropeza C, 2003. *In situ* PCR detection of phytoplasma DNA in embryos from coconut palms with lethal yellowing disease. *Molecular Plant Pathology* 4, 99–108.

Dabek AJ, 1975. The incubation period, rate of transmission and effect on growth of coconut lethal yellowing disease in Jamaica. *Phytopathologische Zeitschrift* 81, 1–9.

Daire X, Clair D, Reinert W, Boudon E, 1997. Detection and differentiation of grapevine yellows phytoplasmas belonging to the elm yellows group and to the stolbur subgroup by PCR amplification of non-ribosomal DNA. *European Journal of Plant Pathology* 103, 507–14.

Deng S, Hiruki C, 1991. Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *Journal of Microbiological Methods* 14, 53–61.

Dery SK, N'cho YP, Sangare A, Arkhurst ED, 1997. Cape St Paul wilt disease: resistance screening and prospects for rehabilitating the coconut industry in Ghana. In: Eden-Green SJ, Ofori F, eds. *Proceedings of an International Workshop on Lethal Yellowing-Like Diseases of Coconut, Elmina, Ghana, 1995*. Chatham, UK: Natural Resources Institute, 147–51.

Dery SK, Nipah JO, Ando-Mensah E, Nuertey BN, Nkansah Poku J, Arthur R, Philippe R, 2005. On-farm evaluation of the coconut hybrid, Malayan yellow dwarf × Vanuatu tall for tolerance to the lethal yellowing disease of coconut in Ghana. *Cord* 21, 50–6.

Dery SK, Nkansah-Poku J, Philippe R, 1999. The history and epidemiology of Cape St. Paul wilt disease of coconut in Ghana. *Journal of the Ghana Science Association* 1, 32–42.

Dollet M, 2002. CIRAD concepts: toward a global research programme on integrated control of lethal yellowing-like phytoplasma diseases of the coconut palm. In: *Proceedings of the Expert Consultation on Sustainable Coconut Production through Control of Lethal Yellowing Disease, Kingston, Jamaica, 2002*. CFC Technical Paper No 18.

Eden-Green SJ, 1997. History, distribution and present status of lethal yellowing-like disease of palms. In: Eden-Green SJ, Ofori F, eds. *Proceedings of an International Workshop on Lethal Yellowing-Like Diseases of Coconut, Elmina, Ghana, 1995*. Chatham, UK: Natural Resources Institute, 9–25.

Harrison NA, Cordova I, Richardson PA, Di Bonito R, 1999. Detection and diagnosis of lethal yellowing. In: Oropeza C, Verdeil CL, Ashburner GR, Cardeña R, Santamaria JM, eds. *Current Advances in Coconut Biotechnology*. Dordrecht, the Netherlands: Kluwer Academic Publishers, 183–96.

Harrison NA, Oropeza C, 1997. Recent studies on detection of lethal yellowing disease phytoplasmas in the Americas. In: Eden-Green SJ, Ofori F, eds. *Proceedings of an International Workshop on Lethal Yellowing-Like Diseases of Coconut, Elmina, Ghana, 1995*. Chatham, UK: Natural Resources Institute, 221–34.

Harrison NA, Richardson PA, Kramer JB, Tsai JH, 1994. Detection of the mycoplasma-like organism associated with lethal yellowing disease of palms in Florida by polymerase chain reaction. *Plant Pathology* 43, 998–1008.

Jones P, 2002. Coconut lethal diseases associated with phytoplasma. In: *Proceedings of the Expert Consultation on Sustainable Coconut Production through Control of Lethal Yellowing Disease, Kingston, Jamaica, 2002*. CFC Technical Paper No 18.

Kirkpatrick BC, 1992. Mycoplasma-like organisms – plant and invertebrate pathogens. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH, eds. *The Prokaryotes*, 2nd edn. New York, USA: Springer-Verlag, 4050–67.

McCoy RE, Howard FW, Tsai JH, Donselman HM, Thomas DL, Basham HG, Atilano RA, Eskafi FM, Britt L, Collins ME, 1983. *Lethal Yellowing of Palms*. Gainesville, Florida, USA: University of Florida Agricultural Experiment Station Bulletin No. 834.

Mpunami AA, Tymon AM, Jones P, Dickinson MJ, 1999. Genetic diversity in the coconut lethal yellowing disease phytoplasmas of East Africa. *Plant Pathology* 48, 109–14.

Nipah JO, 2000. Some characteristics of the Sri Lanka Green Dwarf (SGD): a variety resistant to the Cape Saint Paul wilt disease of coconut. *Journal of the Ghana Science Association* 2, 88–93.

Nkansah-Poku J, Dery SK, Philippe R, Yawson GK, Quaicoe RN, 2003. Studies on artificial induction of Cape Saint Paul wilt disease using putative vectors. *Journal of the Ghana Science Association* 5, 116–22.

Ofori F, Nkansah-Poku J, 1997. Cape St Paul Wilt disease of coconut in Ghana: history of its occurrence and spread. In: *Proceedings of an International Workshop on Lethal Yellowing-Like Diseases of Coconut, 1995*. Chatham, UK: Natural Resources Institute, 27–32.

OPRI, 1998. *Annual Report of the Pathology Division, Coconut Research Programme*. Kusi, Ghana: Oil Palm Research Institute, Council for Scientific and Industrial Research.

Oropeza C, Zizumbo D, 1997. A history of lethal yellowing in Mexico. In: Eden-Green SJ, Ofori F, eds. *Proceedings of an International Workshop on Lethal Yellowing-Like Diseases of Coconut, Elmina, Ghana, 1995*. Chatham, UK: Natural Resources Institute, 69–76.

Romney D, 1983. Brief review of coconut lethal yellowing. *Indian Coconut Journal* 13, 1–8.

Santos GA, Batugal PA, Othman A, Baudouin L, Laboouisse JP, 1994. *Manual on Standardized Research Techniques in Coconut Breeding*. International Plant Genetic Resources Institute & Coconut Genetic Resources Network, 13–6.

Smart CD, Schneider B, Morrer R, Blomquist DJ, Guerra LJ, Harrison NJ, Ahrens U, Lorenze KH, Seemüller E, Kirkpatrick BC, 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-235 rRNA spacer region. *Applied and Environmental Microbiology* 62, 2988–93.

Tymon A, 1995. Etiology and control of Lethal Yellowing-type diseases of coconut palm in Africa. STD III Contract TS*-92-0055. Appendix 4, *Annual Report, 1994*.