MOLECULAR PLANT PATHOLOGY (2003) **4**(2), 99–108

In situ PCR detection of phytoplasma DNA in embryos from coconut palms with lethal yellowing disease

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SUMMARY

DNA of the lethal yellowing (LY) phytoplasma was detected in 13 of 72 embryos from fruits of four diseased Atlantic tall coconut palms by polymerase chain reaction (PCR) assays employing phytoplasma universal rRNA primer pair P1/P7, nested LY group-specific rRNA primer pair 503f/LY16Sr or LY phytoplasma-specific nonribosomal primer pair LYF1/R1. Phytoplasma distribution in sectioned tissues from six PCR positive embryos was determined by *in situ* PCR and digoxigenin-11-deoxy-UTP (Dig) labelling of amplification products. Dig-labeled DNA products detected by colourimetric assay were clearly evident on sections from the same three embryos investigated in detail by *in situ* PCRs employing primer pairs P1/P7 or LYF1/R1. Deposition of blue–green stain on sections as a result of each assay was restricted to areas of the embryos corresponding to the plumule and cells ensheathing it. By comparison, similarly treated embryo sections derived from fruits of a symptomless Atlantic tall coconut palm were consistently devoid of any stain. Presence of phytoplasma DNA in embryo tissues suggests the possible potential for seed transmission which remains to be demonstrated.

INTRODUCTION

Lethal yellowing (LY) is a devastating disease of coconut palm (*Cocos nucifera* L.) and at least 35 other palm species in the Americas (Harrison et al., 1999). Reports of dying coconut palms exhibiting LY-type symptoms date from the 19th century in the Caribbean region (Eden-Green, 1997). During the last three decades, epiphytotics of LY in Jamaica and Florida have been characterized by rapid spread and high losses (McCoy *et al.*, 1983). LY was first recorded in the Yucatan Peninsula of southern Mexico during 1977 (Oropeza and Zizumbo, 1997) and has since spread to Belize (Eden-Green, 1997) and Honduras (Ashburner *et al.*, 1996). A phytoplasma is the known etiological agent of LY which kills palms within a matter of months once symptoms are evident (McCoy *et al.*, 1983). Following a latent (incubation) period estimated between 112 and 262 days (Dabek, 1975), symptoms on the highly susceptible Jamaica tall coconut variety begin with premature nut fall, followed by discolouration (necrosis) of newly emergent inflorescences, a progressive yellowing of successively younger leaves and death of the apical meristem (McCoy *et al.*, 1983). Coconut breeding programs are constantly searching for LY resistant germplasm (Ashburner and Been, 1997) but variations in the LY pathogen may contribute to differential resistance observed among coconut ecotypes in the Americas (Harrison and Oropeza, 1997).

Plant to plant transmission of phytoplasmas occurs primarily during feeding activity by inoculative vector insects, by vegetative propagation of infected plant material, or by graft inoculation (Kirkpatrick, 1992). Although infestation of floral tissues by these obligate, pleomorphic mollicutes has been demonstrated (Clark *et al.*, 1986), it has been generally accepted that seed transmission is unlikely because phloem sieve elements of plants in which phytoplasmas reside lack any direct connection to seeds. Also, yellows disease symptoms often include floral or seed abnormalities (premature nut fall in the case of coconut) thereby preventing production of viable seed once infection has occurred (McCoy *et al.*, 1989). However, a recent report on detection of phytoplasmas in both seed and seedling progeny of alfalfa plants affected by alfalfa witches'-broom disease indicates that seed transmission in certain plant host-phytoplasma pathosystems is indeed possible (Khan *et al.*, 2002). During the last decade, amplification of phytoplasma DNA by polymerase chain reaction (PCR) assays has provided a reliable and specific means of detecting these plant pathogens in both plant and insect hosts (Lee *et al.*, 2000). PCRs employing primer pairs derived from ribosomal or nonribosomal DNA sequences have been used to detect the LY phytoplasma in leaves or inflorescences and in trunk tissues or roots of coconut palm (Harrison *et al.*, 1999). Such studies have also shown that the LY phytoplasma is more readily detected in immature than mature tissues (Cordova, 2000; Escamilla *et al.*, 1995; Harrison *et al.*, 1995), a finding in agreement with earlier etiological investigations of LY employing transmission electron

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microscopy (Thomas, 1979; Thomas and Norris, 1980). Previous reports of the detection of phytoplasma in embryos of fruit from LY-affected coconut palms by PCR (Harrison and Oropeza, 1997; Harrison *et al.*, 1994a) raised questions about the general acceptance of the distribution of phytoplasmas in the cellular environment of host plants. As a result there has been considerable speculation about the possibility that phytoplasmas could be transmitted by seed. This is particularly important for coconut germplasm exchange, where there is much interest in the application of embryo culture for the exchange and conservation of coconut germplasm (Hocher *et al.*, 1999). The FAO/IPGRI Technical Guidelines for safe exchange of coconut germplasm recommend transfer of embryos rather than intact seed to reduce the possibility of inadvertently introducing diseased material into disease-free areas. In this study, we describe the use of *in situ* PCR to localize phytoplasma DNA in embryos from fruits of LY-affected coconut palms as part of a longer term experiment raising embryos through tissue culture. To our knowledge this methodology has not been used previously to visualize prokaryote DNA within a plant embryo.

RESULTS

Phytoplasma detection by PCR

An rDNA product of expected size (about 1.8 kb) was amplified by P1/P7-primed PCR from a single DNA sample out of 72 extracted from embryos of nuts from four LY affected coconut palms. A similar sized product was readily amplified from DNAs of LY-diseased windmill palm (*T. fortunei*) and AY-infected periwinkle (*C. roseus*) included as positive controls in each PCR assay. No product was amplified from each of 10 embryo DNAs from fruits of symptomless coconut palms or DNA extracted from tissues of healthy periwinkle grown from seed. Results of PCR assays are summarized in Table 1. DNA samples from four additional symptomatic palm embryos each yielded a product of expected size (c.1 kb) when subjected to 40 cycles of PCR with LY-specific nonribosomal primer pair LYF1/R1 (Harrison *et al.*, 2002). Reamplification of initial P1/P7-primed products by PCR employing nested rRNA primer pair 503f/LY16Sr (Harrison *et al.*, 1999) consistently yielded an rDNA product of about 930 bp in size from 13 of 72 embryos harvested from LY-affected palms, including embryos previously judged positive by prior PCR assays (Table 2, Fig. 1). A similar size product was amplified from DNA of LY-diseased windmill palm by each of these assays whereas neither assay yielded a product from healthy coconut embryo DNAs or template DNAs derived from AY-infected or healthy periwinkle.

DAPI staining

After longitudinal stem sections from AY phytoplasma-infected or healthy periwinkle plants were treated with the DNA-binding fluorochome DAPI, a light blue fluorescence emitted by nuclei in parenchyma cells adjacent to phloem sieve tube elements was evident when sections were examined by fluorescence microscopy. While the lumen of sieve tube elements of healthy tissues was devoid of stain, streaks of light blue fluorescence indicative of phytoplasma aggregations were observed extending the entire length of some sieve tube elements in sections from AY-infected periwinkle (Fig. 2b). Fluorescing cell nuclei were also apparent in embryo tissue sections. Diffuse patches of bright blue fluorescence, were evident in stained sections from two of six PCR-positive

Table 1 Results of PCR assays on coconut embryo DNA from LY-affected and healthy coconut and control DNAs.

Template DNA	P1/P7	LY16F/LY16R	Nested P1 P7 followed by 503f/LY16Sr
Embryo from LY affected coconut palm	1/72	5/72	13/72
LY affected *T. fortunei*	1/1	1/1	1/1
Embryo from healthy coconut	0/10	0/10	0/10
AY infected periwinkle	1/1	0/1	0/1
Healthy periwinkle	0/1	0/1	0/1

Table 2 Embryos used in this study. Embryos were tested by nested PCR using primers P1/P7 followed by primers 503f/LY16Sr. N = fruits collected but no embryos found.

	Inflorescence Number of embryos tested/number positive						
Palm	A	B	C	D	E	F	G
1(LY)	4/0	4/0	2/1	7/2	7/0	1/0	N
2(LY)	3/1	4/1	2/1	N	N	N	N
3(LY)	2/0	N	4/1	6/2	N	3/0	N
4(LY)	4/1	5/0	5/1	4/1	2/1	3/0	N
Totals	13/2	13/1	13/4	17/5	9/1	7/0	
Healthy	2/0	2/0	2/0	1/0	3/0	N	N

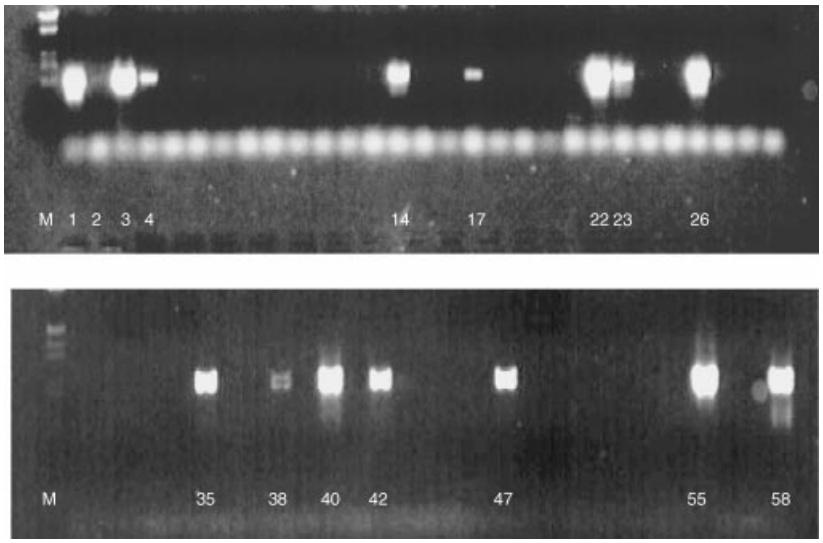

Fig. 1 Agarose gel electrophoresis of PCR-amplified coconut embryo DNA using a nested PCR with universal primers P1/P7 and LY group specific primers 503f/LY16Sr. Only positive lanes are identified: M 1kb ladder, 1, DNA from LY positive coconut palm in Yucatan; 2, water control from first round re-amplified; 3, DNA from LY positive Windmill palm in Florida; 4, 4A1; 14, 4C1; 17, 4D2; 22, 4E1; 23, 2A1; 26, 2B1; 35, 2C2; 38, 3C1; 40, 3D3; 42, 3D5; 47, 1C2; 55, 1D4; 58, 1D7.

embryos examined by this method. In both embryos, fluorescence was confined to an area corresponding to the plumule and one or several layers of adjacent cells (Fig. 2d). No comparable staining of embryo tissues from a symptomless palm was observed (Fig. 2c).

In situ PCR

Deposition of a blue-green stain confined to the lumen of phloem sieve tube elements in AY phytoplasma-infected periwinkle stem sections was seen upon immuno-detection of DNA products resulting from *in situ* PCR using phytoplasma rRNA primer pair P1/P7 (Fig. 3d). By comparison, longitudinal sections of similarly treated tissues from healthy periwinkle stems lacked any sign of colour development (Fig. 3a).

Of the six PCR-positive and 10 PCR negative coconut embryos (five from infected and five from symptomless palms) examined by *in situ* PCR, three of the PCR-positive but none of the negative embryos showed prominent blue-green stain when serial sections were analyzed by *in situ* PCR primed by rRNA primer pair P1/P7 (Fig. 3c). Staining was limited to these same three embryos when additional serial sections from all 16 embryos were reassessed by *in situ* PCR primed by LY-specific nonribosomal primer pair LYF1/R1 (Fig. 4d). On completion of both assays, colour deposition on phytoplasma-positive sections was restricted in all cases to the plumule and surrounding cells (Fig. 4b,c). Signal development was absent from healthy embryo sections used in control reactions (Fig. 4b).

DISCUSSION

The presence of phytoplasma DNA products generated by *in situ* PCR on tissues of three (18%) embryos from highly susceptible Atlantic tall coconut palms with typical LY disease symptoms and absence of product on tissues after assay of all embryos from an apparently healthy coconut palm suggests that the LY agent is able to move through the plant in tissues other than the phloem. The fidelity of the *in situ* PCR assays applied to coconut embryos was supported by our results obtained from parallel investigations of AY-infected and healthy periwinkle tissues (Fig. 3b,c). The latter experimentation clearly demonstrating that the assays were capable of detecting phytoplasma in infected plant tissue that had been fixed, embedded in wax and subjected to the *in situ* PCR protocol. Furthermore, immuno-detection as evidenced by a conspicuous blue-green product and its discrete localization to phloem sieve elements in tissues of AY-diseased and absence of any staining in healthy periwinkle tissues confirmed that the source of template DNA amplified during P1/P7-primed *in situ* PCR was restricted to a phloem-limited phytoplasma target (Fig. 3b,c,4a). The use of nonribosomal primers LYF1/R1, which specifically detect the LY phytoplasma (Harrison *et al*., 1994a, 2002), in addition to the phytoplasma universal rRNA primers P1/P7 further support the accuracy and validity of our results.

Previously, *in situ* PCR has been applied to the detection of viral nucleic acids and transcripts in both animal and plant tissues (Johansen, 1997; Koltai and McKenzie Bird, 2000; Nuovo, 1994). Our investigation of coconut embryos is, as far as we know, the first to apply this technique to detection of phytoplasmas in host plant tissues. The presence of phytoplasmas in coconut embryos conflicts with existing knowledge concerning colonization of host plants by phytoplasmas. Patterns of vascular colonization indicate that phytoplasmas are moved by or move with photosynthate flows in sieve elements from areas of production to areas of utilization in host plants such as the shoot apex, expanding fruits and roots which function as metabolic sinks (Kuske and

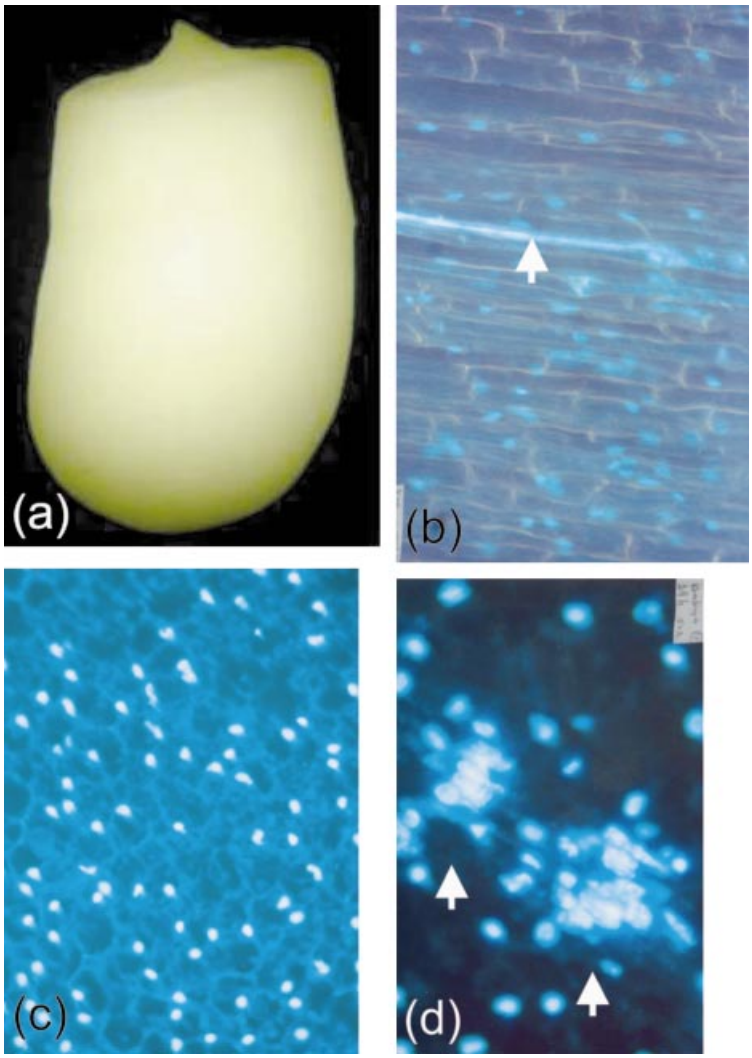

Fig. 2 (A) Coconut embryo height = 7 mm. (B). DAPI staining of AY infected periwinkle stem. Arow points to phloem showing fluorescence along its length. (C) Section of healthy coconut embryo stained by DAPI. Cell nuclei can be seen as fluorescing a light blue. (D) Section of LY affected embryo stained with DAPI. Patches of fluorescence (arrowed) from the region immediately surrounding the plumule.

Kirkpatrick, 1992). As phloem-limited intracellular parasites, an uninterrupted pathway would be required for phytoplasmas to move from the mother plant into the developing ovule or embryo. In coconut, as in other plants, there is no continuous connection between phloem sieve elements of the inflorescence and the developing embryo in fruit (Menon and Pandalai, 1960).

Although confinement of phytoplasmas to the phloem in plants is generally accepted by plant pathologists, nevertheless, it remains a concept of some conjecture in light of past studies in which evidence of phytoplasmas in phloem parenchyma and cell types other than phloem sieve elements was reported (Amici and Favali, 1972; Sears and Klomparens, 1989; Siller *et al.*, 1987). McCoy (1979) considered phytoplasma infestation of parenchyma cells to be unlikely due in part to the unfavorably low osmotic potential of these cells as compared to the cellular environment of sieve elements and because phytoplasmas were considered too large to move though parenchyma plasmodesmata; a hypothesis supported by ultrafiltration studies.

In contrast to plants, no such restriction on phytoplasma distribution is evident in host insects as they have been found in most major organs of infected vector insects (Alma *et al.*, 1997). For such extensive colonization to occur, ingested phytoplasmas must be capable of penetrating and passing though the epithelial cells of the midgut in order to enter, multiply and disseminate within the circulatory system of their respective vectors. It has also been established that phytoplasmas must traverse the membrane of the posterior acinar cells of salivary glands before vectors are capable of transmitting them while feeding on plants (Kirkpatrick, 1992). Ultrastrucutural analysis of both mid-gut epithelia and salivary glands of *Spiroplasma citri*-infected beet leafhoppers (*Circulifer tenellus*) revealed spiroplasmas in peripheral membrane-bound cytoplasmic vesicles or within membranous pockets formed by invagination of the plasmalemma, suggesting that spiroplasma entry into insect cells occurs by receptor mediated endocytosis (Kwon *et al.*, 1999). Past reports of phytoplasmas in phloem parenchyma (Amici and Favali, 1972; Siller *et al.*,

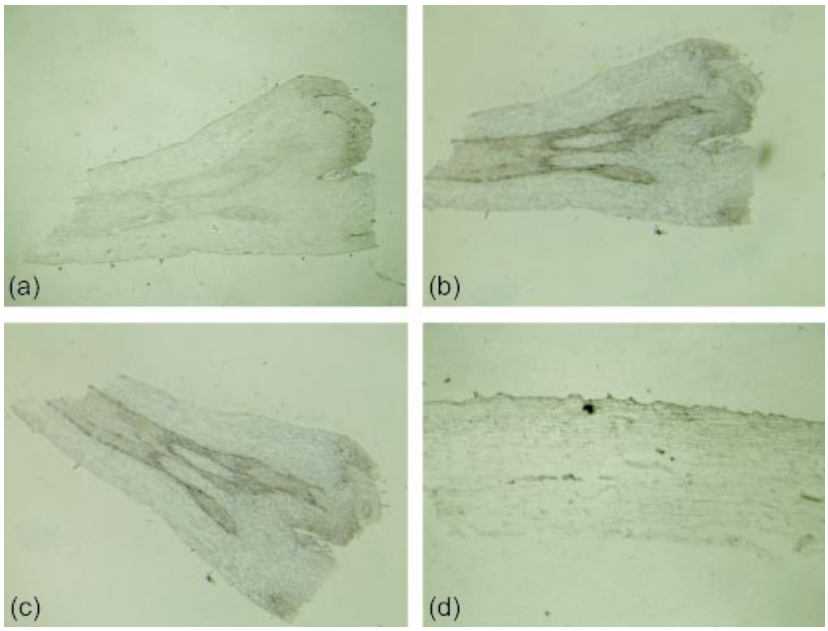

Fig. 3 Sections of periwinkle stem after *in situ* PCR. (A) Negative control section of AY-infected periwinkle, PCR done without primers. (B) and (C) Sections of stem from periwinkle infected by AY phytoplasma after *in situ* PCR using primers P1/P7. Deposition of stain following immuno detection can be seen following the phloem cells of the vascular bundles. (D) Negative control section of healthy periwinkle stem after *in situ* PCR using primers P1/P7.

1987) or in companion cells (Sears and Klomparens, 1989) of host plants have been largely dismissed as misidentifications of immature phloem as parenchyma cells or as erroneous identification of cytoplasmic vesicles of host origin as phytoplasma (McCoy, 1979).

Prompted by past reports on PCR detection of phytoplasma DNA in embryos from seed of LY-diseased coconut palms (Harrison and Oropeza, 1997; Harrison *et al*., 1994a), we have since used transmission electron microscopy (TEM) to search for phytoplasmas in embryo tissues but were unable to demonstrate their presence beyond doubt (unpublished data). Because phytoplasmas are pleomorphic, possess few distinctive features and are present in host plant tissues often in low titer, it can be difficult to detect them by TEM (Thomas, 1979); or to differentiate them from other membrane-bound bodies that occur in sieve elements of healthy plants or plants affected by virus diseases (McCoy, 1979). In such situations, positive identifications have been facilitated by combining microscopy with immunolabeling of phytoplasmas (Jiang *et al*., 1989; Lherminier *et al*., 1990). Because of difficulties in acquiring suitably purified phytoplasmas from palm tissues in sufficient quantity for use as antigen, antibodies have not been raised against the LY agent thus precluding immunocytochemistry to complement our TEM analysis of coconut embryo tissues.

Most recently, *in situ* hybridization employing various phytoplasma-specific oligonucleotide probes and reporter systems has been used as an alternative approach to detect, identify and examine phytoplasma distribution in host tissues (Deng and Hiruki, 1991a; Lherminier *et al*., 1999; Webb *et al*., 1999). By combining *in situ* hybridization with TEM, Lherminier *et al*. (1999) found that all previously described morphotypes of phytoplasmas including senescent condensed forms were detected in sieve elements of infected plants by this method.

Recently, Kawakita *et al*. (2000) used a conventional PCR assay to detect and identify mulberry dwarf (MD) phytoplasmas in ovaries and testes of its vector leafhopper *Hishimonoides sellatiformis* supporting their TEM observations of the MD agent in these tissues. The possibility of transovarial transmission of phytoplasmas suggested by these and earlier results (Alma *et al*., 1997) was augmented by PCR amplification of MD phytoplasma DNA from both eggs and newly hatched nymphs of inoculative *H. sellatiformis*. Notably, until these reports it was widely believed that transovarial transmission of phytoplasmas did not occur.

Since few embryos collected from LY-diseased coconut palms were found to contain detectable phytoplasma DNA in the present study, this observation suggests that the timing of infection relative to the developmental stage of the inflorescence or fruit is important for phytoplasma infestation to occur. At palm maturity, the developing inflorescences are present in the young leaf axils of the apical bud, and so, the phytoplasma could theoretically reach these vulnerable tissues at any time after infection. A progressive discolouration (necrosis) of unemerged or newly emergent inflorescences is an early stage symptom, invariably associated with LY disease, at which time nondiscoloured parts of the flower spikes contain phytoplasma that are detectable by TEM (Thomas and Norris, 1980) or PCR (Harrison *et al*., 1995). These inflorescences wither and die without setting fruit. The time from pollination/fertilization of the ovule to fruit maturity in coconut palm varies between 11 and 13 months according to ecotype (Whitehead, 1965) and is of similar duration to the

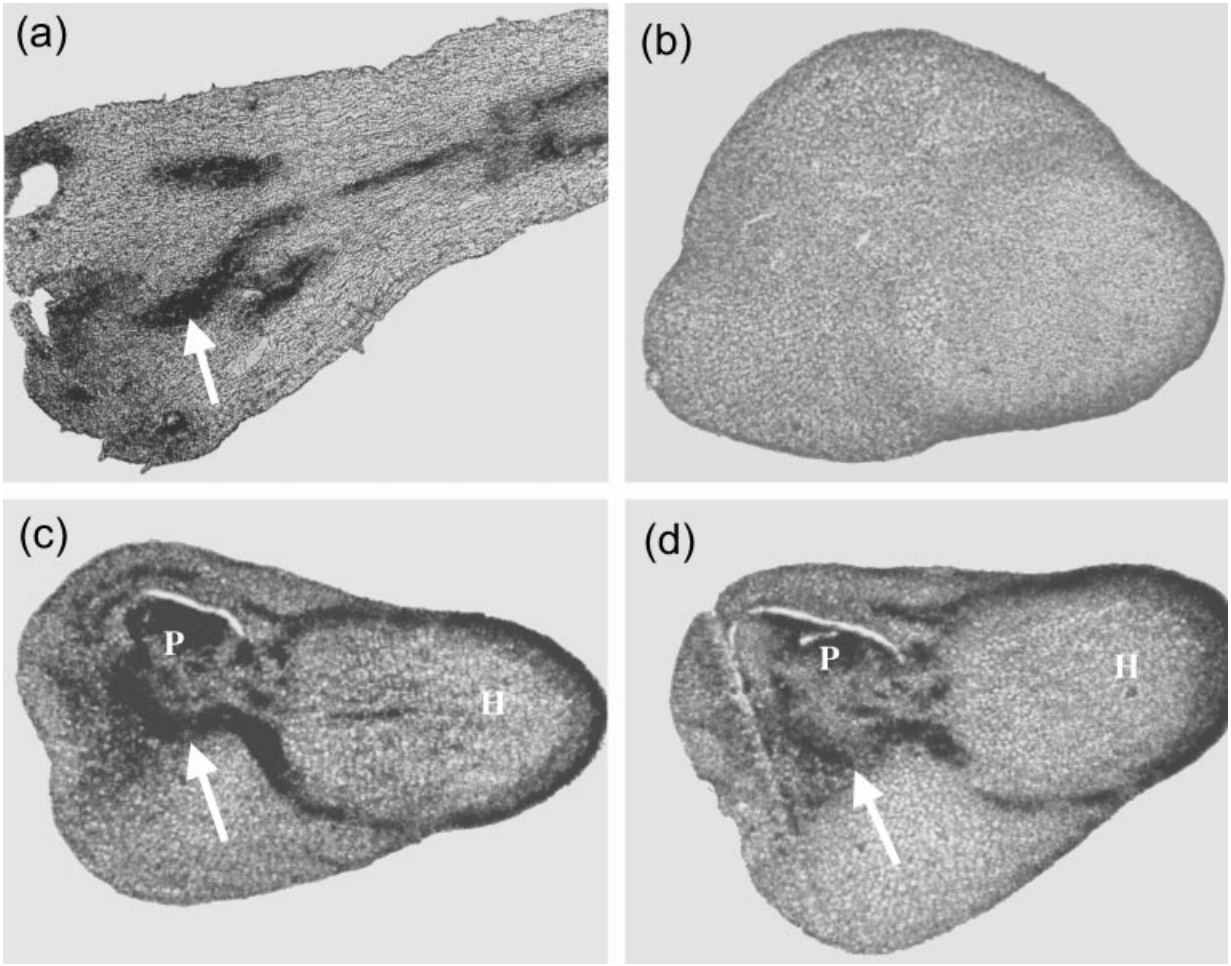

Fig. 4 Detection of LY phytoplasmas by *in situ* PCR in sections from coconut embryos and periwinkle stem tissues. (A) Periwinkle stem after *in situ* PCR using universal phytoplasma primers P1/P7. Arrow indicates deposition of stain in node region of stem extending along the vascular bundles into the internode region. (B) Healthy coconut embryo after *in situ* PCR using LY Group specific primers 503f/LY16Sr, no staining is evident. (C) Embryo from LY-affected coconut palm after *in situ* PCR using LY Group specific primers 503f/LY16Sr. Arrow indicates deposition of stain in the region surrounding the plumule (P) and the cells surrounding the developing haustorium (H). (D) Embryo from LY affected coconut palm after *in situ* PCR using LY phytoplasma specific primer pair LYF1/R1. Arrow indicates deposition of stain in the region surrounding the plumule (P) and the cells surrounding the developing haustorium (H).

estimated incubation (presymptomatic) phase for LY on the Atlantic tall coconut ecotype (Dabek, 1975). Thus embryo infestation by phytoplasma is probably limited to developing fruit set before the palms contracted LY.

Presence of phytoplasma DNA in coconut embryos raises the possibility of seed transmission of LY disease in this palm species. One important limitation to phytoplasma detection by DNA-based methods such as *in situ* PCR is that none of these methods are capable of selectively discriminating viable from nonviable phytoplasma. Instead an appropriate and sensitive bioassay will be necessary to unequivocally determine the fate of phytoplasma-positive seed. This might be accomplished through PCR analysis of emergent sprouts upon germination of fruits from LY-diseased coconut palms or alternatively by germination of excised embryos using *in vitro* methods (Ashburner *et al.*, 1995) and analysis of resulting plantlets. Our preliminary attempts at such bioassays have been complicated by the fact that very few fruit harbor phytoplasma-positive embryos; a lack of any apparent morphological differences between infected and uninfected fruit or embryos; and a markedly diminished viability of fruit from diseased palms. The need for appreciable numbers of seed to offset these considerations preclude seed transmission studies in LY-endemic areas where susceptible coconut ecotypes lost to LY have been largely replaced by resistant ecotypes and hybrids among which disease incidence is usually low. In these areas, establishment of experimental plantings of LY-susceptible coconut ecotypes to provide a source of fruit for bioassay is also impractical, since the disease usually eliminates most of these

palms before they reach maturity. For these reasons, future progress toward clearly resolving the issue of seed transmission of phytoplasma diseases will require study of a more amenable herbaceous plant pathosystem such as alfalfa witches'-broom (Khan *et al.*, 2002) rather than an intractable woody perennial plant host such as coconut palm.

EXPERIMENTAL PROCEDURES

Plant samples

Fruits still attached to the infructescence were collected from four Atlantic tall coconut palms displaying typical LY disease symptoms (McCoy *et al.*, 1983) on a plantation in Yucatan, Mexico. For comparative purposes, mature fruits were also harvested from a symptomless, presumably healthy, Atlantic tall coconut palm. Once this species reaches maturity it produces a new inflorescence approximately once a month, thus inflorescences were categorized from A (oldest) to G (youngest) (Table 2). Fruits were returned to the laboratory where embryos (Fig. 2a) were excised from them by the method of Rillo (1999) and stored at 4 °C until used. Periwinkle (*Catharanthus roseus* G. Don) singly infected with aster yellows phytoplasma (European strain, provided by M. Clark, Horticulture Research International, UK) was maintained in an insect-proof greenhouse as were seed-grown, healthy periwinkle plants. DNA derived from immature leaf bases of a symptomatic Windmill palm (*Trachycarpus fortunei* (Hook) H. Wendl.) on the grounds at the University of Florida's Research and Education Center, Fort Lauderdale, FL served as a source of LY phytoplasma DNA for comparison.

DNA extraction

Each coconut embryo was split longitudinally into halves using a clean razor blade. One half of each embryo was ground in a 1.5-mL centrifuge tube using a disposable pestle. DNA was extracted from the resulting tissue slurry by the method of Doyle and Doyle (1990). Ethanol-precipitated nucleic acids were dried briefly, resuspended in 100 μL of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8) and incubated with RNAse for 1 h at 37 °C. Aliquots of final embryo DNA preparations were used as template for PCR.

Amplifications were performed in 25 μL reaction volumes each containing 1 μL of embryo DNA, 50 ng of each primer, 125 μM of each dNTP, 1 U Taq DNA polymerase and standard PCR buffer containing 1.5 mM $MgCl_2$. PCR was performed for 35 cycles in a programmable Omnigene thermocycler (Hybaid, Ashford, UK) employing phytoplasma-universal rRNA primer pair P1 (Deng and Hiruki, 1991b) and P7 (Smart *et al.*, 1996) and previously reported thermal cycling conditions (Harrison *et al.*, 2001). Products resulting from initial P1/P7-primed PCR were diluted 1 : 40 with sterile ultrapure water and reamplified for 30 cycles using nested 16S rRNA primer pair 503f (Harrison *et al.*, 2001) and LY16Sr (Harrison *et al.*, 2002). For nested PCR the following thermal cycling parameters were used: denaturation for 30 s (2 min 30 s for first cycle) at 94 °C; annealing at 60 °C for 50 s and extension at 72 °C for 1 min 20 s (10 min for final cycle). All embryo DNAs were reassessed by PCR (40 cycles) employing LY phytoplasma-specific, nonribosomal primer LYF1 and LYR1, as previously described (Harrison *et al.*, 1994b). Aliquots (5 μL) of each final reaction mixture were electrophoresed though 1% agarose gels, stained with ethidium bromide, visualized by UV transillumination and photographed.

Preparation of plant tissues for *in situ* PCR

Remaining halves of coconut embryos, as well as stem pieces (2 × 5 mm) from AY-infected or healthy periwinkle plants included for comparative purposes, were immersed in chilled (4 °C) fixative (4% paraformaldehyde, pH 7.2) for 24 h. Samples were washed with three changes of saline (145 mM NaCl) solution and then dehydrated by serial transfer to a graded ethanol series (30%, 50%, 75%, 85% and 90%; 1 min per step) diluted with saline. Following immersion in two changes of absolute ethanol, samples were transferred to an absolute ethanol-Histoclear (Sigma-Aldrich Co., Dorset, UK) solution (1 : 1 v/v) for 2 h and then into Histoclear only for 2 h. After two changes of Histoclear, samples were transferred to a 60% solution of Paraplast Plus wax (Sigma) diluted with Histoclear at 40 °C for about 16 h before transfer to molten (60 °C) Paraplast which was changed twice a day for 3 days. Infiltrated samples were sealed in plastic molds of fresh molten Paraplast by plunging the molds into cold water to quickly harden the wax (Schwarzacher and Heslop-Harrison, 2000).

Longitudinal sections of tissue about 10 μm in thickness were cut from each wax-embedded sample on a rotary microtome (Model 820 Spencer Microtome, American Optical Co., New York, USA). Sections were collected with a fine paintbrush and floated on a drop of sterile distilled water on glass microscope slides precoated with a thin film of Polysene (BDH Laboratories, Poole, UK). Slides were then placed on a hot plate at 42 °C for about 5 min to expand the sections. After draining off excess water, the slides were dried overnight in an oven at 42 °C.

DAPI staining

Tissue sections were dewaxed in two changes (15 min each) of Histoclear prior to immersion in an aqueous (1 μg/mL) solution of DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, hydrate) (Sigma) for 30 min at 25 °C (Seemüller, 1976). Treated sections were rinsed in an aqueous 2.5 mM $MgCl_2$ solution, mounted in Citifluor (Agar Scientific, Stansted, UK) and viewed using a Leitz Othoplan microscope equipped with a UV epi-illuminator.

In situ PCR

The methods used for preparing tissue sections were essentially those of Schwarzacher and Heslop-Harrison (2000). Briefly, sections were dewaxed as previously described, rehydrated in a graded, 100%, 90%, 85%, 75%, 50% and 30% ethanol series (1 min per step) and then immersed for 10 min in a 1% HCl solution containing 0.25% Triton X-100. Sections were rinsed for 5 min in phosphate-buffered saline (PBS), pH 7.2 before reimmersion for 2 min in a fresh 1% HCl, 0.25% Triton X-100 solution. Sections were digested by an initial incubation in proteinase-K (5 μg/mL) solution for 15 min at 37 °C and then in a Pronase E (10 μg/mL) solution for 10 min at 25 °C. The digestion was completed by immersion of sections in 0.2% glycine. In order to rebuild cross-linkages lost during proteolytic digestion, sections were placed in 4% paraformaldehyde for 20 min then washed with two changes (15 min each) of PBS and once for 5 min in sterile distilled water. Sections were then dehydrated in a graded ethanol series (30%, 50%, 75%, 85% and 90%; 2 min per step) and finally air-dried for 2 h at 25 °C.

For *in situ* PCR, the following reaction mixture components were used: 2.0 mM $MgCl_2$, 200 μM of each dNTP, 10 μM digoxigenin-11-deoxy-UTP (Roche Diagnostics, Lewes, UK), and 100 ng of each primer in PCR buffer II (Perkin Elmer, Beaconsfield, UK). The mixture was heated to 70 °C for 10 min before adding 2 U of AmpliTaq Gold DNA polymerase (Perkin Elmer). To avoid spreading and evaporation of the reaction mixture, each tissue section was confined on a microscope slide by an *in situ* Frame (Eppendorf UK Ltd, Cambridge, UK) before adding 60 μL of reaction mixture to the surface of the section. The frame was then covered with polyester film. Amplifications were performed for 30 or 40 cycles in an Omnislide thermocycler (Hybaid) using the following thermal cycling conditions: denaturation at 94 °C for 1 min (5 min at 95 °C for first cycle), annealing at 55 °C for 1.5 min (primer pair P1/P7) or at 53 °C for 50 s (primer pair LYF1/R1) and extension at 72 °C for 3 min (10 min for final cycle).

Immuno-detection of PCR products

On completion of each PCR, the *in situ* frame was carefully removed and tissue sections were washed by immersing the microscope slides in two changes (15 min each) of TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) buffer to eliminate unbound nucleotides. After sections were air-dried at 25 °C they were incubated in two changes (1 h each) of freshly prepared 1% blocking buffer (Roche Diagnostics) in TBS, pH 7.5. Treated sections were air-dried at 25 °C and a ring was drawn around individual sections on microscope slides using hydrophobic ink. Each section was incubated in 100 μL of antidigoxigenin alkaline phosphatase-conjugated monoclonal antibody (antidig-AP-Mab) (Roche Diagnostics) diluted 1 : 500 with blocking buffer for 2 h in a humid chamber and then washed with two changes (15 min each) of a magnesium buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). Finally, 100 μL of NBT/BCIP substrate solution (Roche Diagnostics) prepared according to the manufacturer's instructions was added to the surface of each section and slides were once again placed in a humid chamber and incubated in a dark room. Sections were examined for evidence of substrate colour development by light microscopy at 10 min intervals. Reactions were stopped by immersion of sections in distilled water from 25 to 60 min after adding the substrate. Positive reactions were indicated by deposition of an insoluble blue-green stain on the surface of sections which were mounted in Aquamount (BDH Laboratories), viewed with a Leitz Orthoplan microscope and photographed. Negative controls were always included during each *in situ* assay in order to identify potential false positive or false negative results. These consisted of both embryos from symptomless coconut and tissues treated with reaction mixtures in which either the DNA polymerase, primer pair or digoxigenin-11-deoxy-UTP was omitted.

ACKNOWLEDGEMENTS

We thank Rothamsted International for the award of a Rothamsted International Fellowship to I. Cordova. Work in the UK was done under DEFRA licence number PHL37C/3564.

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