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Phytoplasma Associated with Yellowing Disease of *Washingtonia* sp. in Kuwait

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Yellowing disease of palms caused by phytoplasma is spreading in the Arabian Gulf region. Surveys were conducted to determine the occurrence of the disease. Electron and fluorescence microscopy, and polymerase chain reaction (PCR) techniques were used to detect the phytoplasma associated with the yellowing disease of ornamental palm *Washingtonia* sp. grown in Kuwait. An accumulation of phytoplasmal DNA was observed by fluorescence microscopy in phloem tissues of diseased palms. Electron microscopy showed that phytoplasma cells were primarily confined to the phloem-sieve elements of tissue samples collected from infected mature palms in the field. The pathogen was identified on the basis of molecular analysis using universal and specific nested primers in PCR amplifications. Prokaryotic 16S rDNA gene was detected in amplified PCR products. Nested PCR resulted in DNA amplification of 1.2 kbp fragment. This is the first report of a phytoplasmal rDNA gene identified from the putative causal pathogen of yellows in ornamental palms in the Arabian Gulf region.

Keywords **:** nested PCR, ornamental palms, phytoplasma, TEM.

Doi et al. (1967) discovered pleomorphic prokaryotic mycoplasma-like organisms (MLOs) in the phloem cells of four plants affected by different yellows-type diseases; which is a group of diseases that show similar yellowing symptoms. A major limitation to acquire important etiological and epidemiological information concerning MLOs, (Krieg and Holt, 1984) during the last two decades has been the lack of rapid, sensitive, and specific means for pathogen detection and disease diagnosis. The MLOs have not been cultured, consequently, diagnosis has relied primarily on symptomatology, florescence and electron microscopy, and recent molecular techniques. The change in terminology from MLOs to phytoplasma in 1994 by Sears and Kirkpatrick reflected new knowledge about plant infecting pleomorphic Mollicutes. Studies of DNA homology in the highly conserved genes encoding ribosomal RNA and ribosomal protein have shown that phytoplasmas comprise a coherent set distinct from all other prokaryotes (Gundersen et al., 1994). Several groups of phytoplasmas have been differentiated on the basis of nucleotide sequence variations in 16S ribosomal RNA genes (Seemuller et al., 1994). This differentiation is supported by sequence homology in ribosomal protein genes and in random parts of the phytoplasma genome (Gundersen et al., 1994). Substantial progress has been made in recent years toward improved detection and diagnosis of phytoplasma-associated diseases by the application of both hybridoma and PCR technology. There is little information available about yellowing diseases of palms in Kuwait. The disease is a serious problem that might get out of control and spread throughout the Arabian Gulf region. The purpose of this study was to detect and identify the putative causal agent associated with yellowing disease of ornamental palms in Kuwait.

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Materials and Methods

Sample Collection. Leaf and root samples were collected from young and mature ornamental palms *Washingtonia* sp. showing yellowing symptoms. Samples were collected from the following fields and nurseries in the State of Kuwait: Omariya (15 samples), Wafra (25 samples), Rabia (15 samples), Keifan (20 samples), and Sabahiya and Mishref (10 samples). In addition, 3 healthy samples showing no yellowing symptoms were collected from each area as a control. All samples were kept on ice or immediately frozen in liquid nitrogen for analysis.

Fluorescence Microscopy. Preliminary examinations of both healthy and infected samples were conducted using fluorescence microscopy. Sections of about 30-50 μm thick were made by cryotome as employed by Davis et al. (1996) and stained with DAPI (4',6-diamidino-2-phenylindole) (Sigma Chemical Co., St. Louis, MO) according to Hiruki and Chen (1981). The stained sections were later rinsed in the buffer and mounted in 87% glycerol according to Nienhaus et al. (1982). Another set of freeze cryotome sections were stained with 20 μg/ml acridine orange (Sigma), pH 7.5, for 3 min.

Transmission Electron Microscopy (TEM). Infected and healthy leaf samples of *Washingtonia* sp. were sliced, fixed in 5% glutaraldehyde, postfixed in 1% osmium tetroxide, and dehydrated in an automatic tissue processor (Leica, Reichert-lynx, Vienna, Austria). The samples were then soaked in different ratios

of propylene oxide: Spurr resin mixture (Spurr, 1969), treated with pure Spurr resin and finally embedded in capsules which were initially cured in a vacuum oven (Isotemp, Fisher, Fair Lawn, NJ). Ultra-thin sections (~80 nm) were cut, transferred onto 150 mesh copper grids, stained with uranyl acetate followed by lead citrate and then examined by transmission electron microscopy (JEOL's JEM-1200 EX II) at the Electron Microscopy Unit, Kuwait University (Thomas, 1979).

Ultrathin Sections of Purified Phytoplasma. A modification of the technique of Sinha (1974) was employed for the purification of phytoplasma cells. Two hundred grams of infected leaf tissue were cut in 10 ml of chilled isolation medium, disrupted with a Sorvall Omni-mixer and ground with a mortar and pestle. The sap was squeezed through cheesecloth, and the volume was adjusted to 800 ml with isolation medium, then centrifuged at 1,500 × g for 8 min. The supernatant was recentrifuged at 35,000 × g for 30 min. The pellet was resuspended in the suspending medium, pooled and recentrifuged as above. Healthy plant tissues were treated similarly and used as controls. One ml of the preparation was added to the top of each discontinuous percoll gradient tube, and centrifuged at 30,000 × g for 60 min at 4°C in a fixed-angle Type 30 rotor (Beckman, Palo Alto, CA). The fractions were resuspended and recentrifuged at 35,000 × g for 2 h.

Pellets containing phytoplasma cells were fixed in 2% gultaraldehyde (Sinha, 1974), centrifuged at 15,000 × g for 30 min, and embedded in 1% agarose. The samples were postfixed in buffered 1% osmium tetraoxide, washed three times and embedded in Spurr's resin (Spurr, 1969, Waters and Hunt, 1980). Ultrathin sections (~80 nm) were cut, stained with uranyl acetate and lead citrate.

Freeze Fracturing for Scanning Electron Microscopy (SEM). The modified technique of Humphreys et al. (1974) was used for the preparation of freeze fractured samples. Tissues prepared as described above were taken from absolute ethanol and placed on copper support disks, then frozen in liquid nitrogen. Samples that had been dried to their critical point were mounted and coated with gold under vacuum on a rotary stage, then examined in a JEOL's JSM-6300 SEM.

Total DNA Extraction. Total DNA was extracted using the miniprep procedure of Doyle and Doyle (1990), except that the tissues were not frozen in liquid nitrogen. Instead, 5 g fresh palm heart tissues (Gomar in Arabic) were directly ground to a paste in a cold mortar and pistol with acid-washed sea sand, and 15 ml of cytamole trimethyl amide borate buffer preheated at 65°C, (2% w/v of cytamole amide borate buffer, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, and 100 mM Tris-HCl, pH 8.0). After incubation at 60°C for 30 min, the lysate was cooled to room temperature, then extracted with an equal volume of chloroform/isoamyl alcohol at 24 : 1(v/v). The mixture was centrifuged at 20,000 × g for 10 min then the aqueous phase was collected by a Pasteur pipette. One volume of isopropanol and 0.1 volume of 3 M sodium acetate pH 5.2 were added to precipitate the nucleic acids. Large cobwebs of nucleic acids were then spooled out with a glass hook. Alternatively, the mixture was chilled at -20°C for at least 2 h, then centrifuged at 20,000 × g for 10 min to precipitate the nucleic acid. Pellets were each rinsed in 0.5 ml of 80% (v/v) ethanol and allowed to air-dry. Pellets were then dissolved in 2 ml of 1x TE buffer pH 8.0 containing RNase at a concentration of 10 µg /ml, and incubated at 37°C for 1h. One-fourth volume of 5 M NaCl and 2.5 volumes of cold 95% (v/v) ethanol were added to each tube to precipitate the DNA as described above. Pellets were again rinsed, dried, and dissolved in 1 ml of 1x TE buffer pH 8.0, and stored at -20°C.

Universal Detection of 16S rDNA using PCR. DNA extracts derived from infected and healthy palm tissues as well as positive control DNAs from phytoplasma infecting wind mill palms (kindly provided by Dr. Phil Jones, Integrated Approach to Crop Protection Research, Rothamsted, UK), were analyzed initially by direct PCR using primer pair R16mF2 (5'-CATGCAAGTCGAACGA-3') and R16mR1 (5'-CTTAACCCCAATCATCGAC-3') (Lee et al., 1993). The universal primers were designed for amplifying DNA fragments from nucleotide position # 53 to #1487 on the 16S rDNA sequence. PCR assays were performed with 50 ng of total nucleic acids or total DNA after RNase treatment and 10 µl of a mixture of each deoxynucleotide triphosphate (dNTPs) at 200 µM. Primer pairs at 40 pmol/µl were added to 5 µl of 10x reaction buffer (1.5 mM $MgCl_2$, 10 mM Tris pH 8.3, 50 mM KCl), 5 µl $MgCl_2$ solution and 0.25 µl of DNA *Taq* polymerase (about 12.5 units, Perkin Elmer, USA) just before running PCR thermal cycles. A final volume of 50 µl of the PCR reaction mix was made up with dH_2O. DNA samples were amplified in a Perkin-Elmer Thermal Cycler (Montasser et al., 1999). Thirty five cycles were employed with an initial denaturing step of 94°C for 1 min, annealing temperature at 60°C for 2 min and extension at 72°C for 3 min, then a final extension step at 72°C for 10 min.

Nested PCR Assays. PCR products previously amplified by using universal primers were employed as templates for nested PCR. Nested primer pairs of R16F2n (5'-GAAA CGACTGCTAAGACTGG-3'), and R16R2 (5'-GACGGGCGGTGTGTACAAACCCCG-3'), that were designed to amplify DNA segments from nucleotide position # 152 to #1397 on the phytoplasmal 16S rDNA sequence (Lim and Sears, 1989) were used. The reaction mixture of 50 µl contained 200 µM of dNTPs, 40 pmols of each primer R16F2n/R16R2, 0.25 µl DNA *Taq* polymerase (about 12.5 units, Perkin Elmer, USA), 5 µl 10x PCR buffer was used with the DNA samples which were diluted to 1:40 with sterile molecular grade water (Sigma chemical Co.). DNA samples were amplified for 35 cycles under the thermal cycling conditions of initial denaturing step at 94° C for 1 min, annealing temperature at 55°C for 2 min and extension at 72°C for 3 min, with a final extension step at 72°C for 10 min. Polyacrylamide gel electrophoresis (PAGE) as well as low melting agarose gel (1% agarose) were employed as described by Montasser et al. (1999). Gels were stained with ethidium bromide or silver nitrate.

Pathogen specific primer pair LY 1 (5'-CATATTTTATTTCCTTTGCAATCTG-3'), and LY2 (5'-TCGTTTTGATAATCTTTCATTTGAC-3') designed for genomic DNA of lethal yellows isolated from a windmill palm (*Trachycarpus fortunei*) was used. The designed primers that enable specific detection of the pathogen of lethal yellows were used according to Harrison et al. (1994). PCR was performed in a 50 µl reaction mixture containing 50 ng of each primer. Forty cycles of PCR were required

under conditions of denaturing step at 94°C for 60 sec, annealing at 53°C for 50 sec and primer extension at 70°C for 80 sec were performed in the PCR machine as described previously. Reaction mixtures containing DNA extracted from healthy palm tissues, or sterile distilled water substituted for template DNA, was used as a negative control that were employed in each experiment for all PCR amplifications.

Restriction Fragment Length Polymorphism (RFLP) Analysis. Nested-PCR amplified 16S rDNA products were digested with *Taq*I at 65°C for a minimum of 8-16 h. Digests were electrophoresed through either 1% NuSieve GTG agarose gels or 5% non-denaturing polyacrylamide gels. The running buffer used was as described by Montasser et al. (1999). Digested 16S rDNA fragment profiles were stained by either ethidium bromide or silver staining. The classification system reported by Lee et al. (1998) was used as a reference for this study.

Results

Disease Incidence and Symptomatology. All samples collected from ornamental palms *Washingtonia* sp. showing typical yellowing symptoms were infected with phytoplasma; and the results indicated its prevalence in all visited locations in the State of Kuwait. We could not detect any amount of phytoplasma in healthy-looking palms without yellowing symptoms. The early disease symptoms were marked by partial yellowing starting at the lower leaf margins with progressive discoloration, ending with a complete bright yellowing of the leaves and subsequent wilting and drooping of leaves parallel to the trunk. The leaves then turned grayish brown in color and tended to fall.

Fluorescence Microscopy. Presumptive phytoplasmal DNA was detected using DAPI. The DNA appeared as bluish-white fluorescent specks or aggregates in sieve tubes when viewed with a fluorescence microscope. Fluorescence was present in longitudinal sections of infected, but not healthy, roots of *Washingtonia* sp. (Fig. 1). The moderately fluorescent spots observed in the healthy tissue can be attributed to the nuclear DNA of the host cells. The distribution and intensity of the fluorescent spots were proportional to the degree of severity of the external symptoms on the palms. Infected vascular bundles stained with acridine orange exhibited a bright yellow fluorescence. Similar staining patterns were not observed in sections obtained from healthy palms.

Electron Microscopy. Ultra-thin sections of infected tissues showed the presence of characteristic pleomorphic bodies, such as budding forms, filamentous branching forms, small or large spherical bodies containing densely stained cytoplasm and closely packed ribosome-like particles or granules, and large bodies with bleb or protrusion (Fig. 2). As the numbers of phytoplasma structures increased, they became well distributed throughout the cell lumen as seen in diseased palms. The size of spherical phytoplasma cells ranged from 80-1000 nm in diameter. The pleomorphic forms of phytoplasma seen in infected plant tissues were absent in healthy tissues, indicating that phytoplasma was associated with yellowing disease.

Purification of Phytoplasma. Micrographs of purified

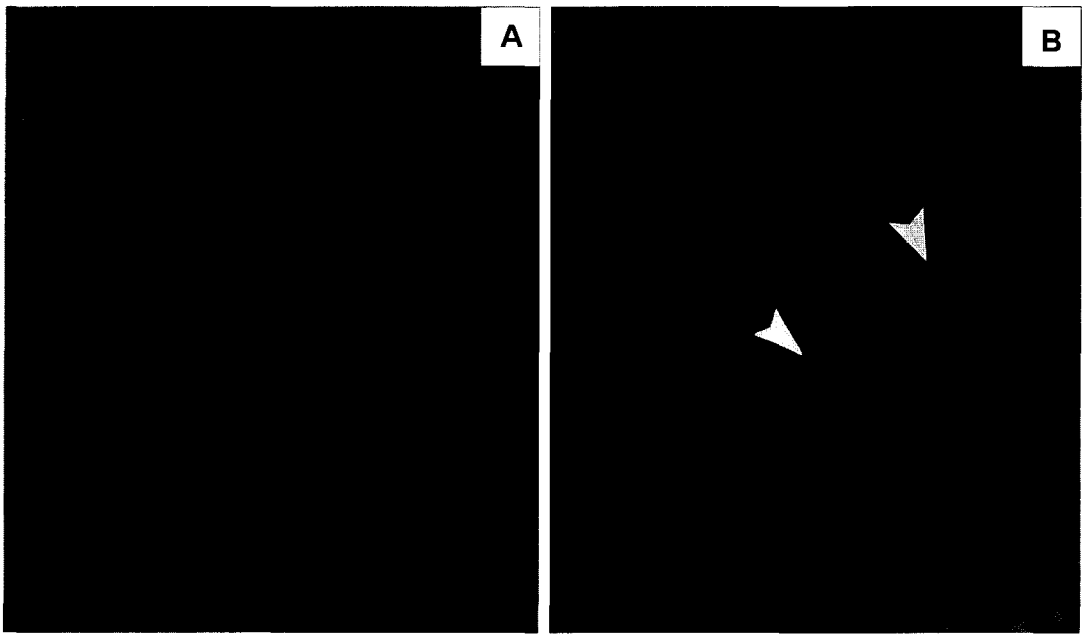

Fig. 1. Fluorescent microscopy of vascular tissues in longitudinal sections of roots of *Washingtonia* sp. palms. (**A**) Healthy palms and (**B**) infected palms (mag. 40X).

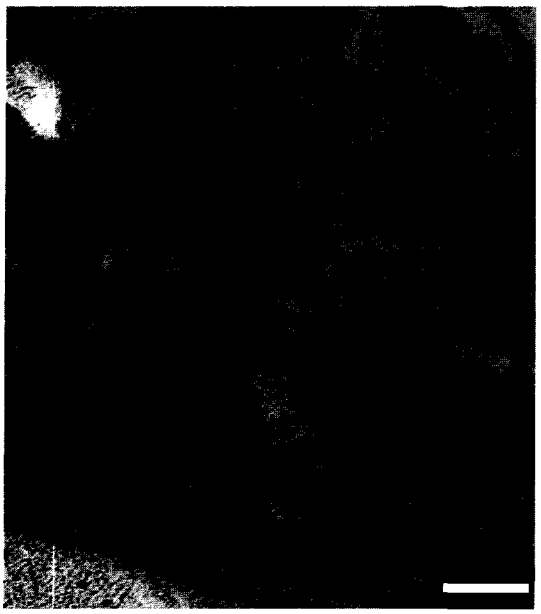

Fig. 2. TEM micrograph of phytoplasma cells *in situ* in phloem tissue of diseased *Washingtonia* sp., bar = 100 nm.

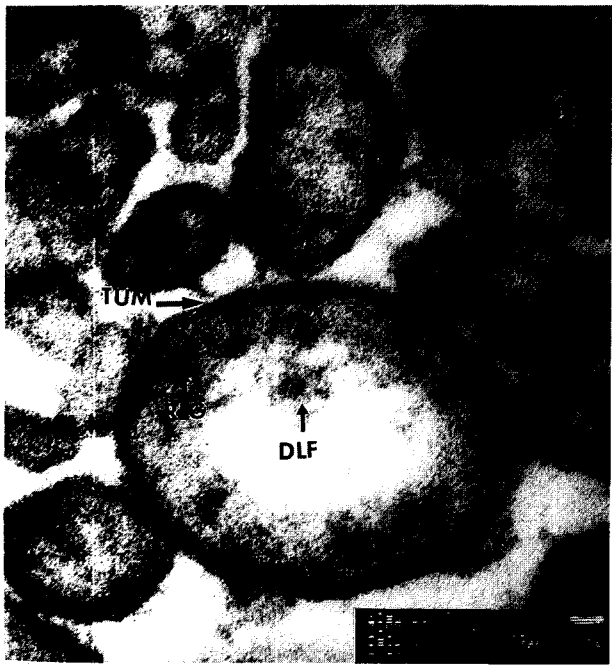

Fig. 3. TEM micrograph of phytoplasma cells purified by density gradient technique from infected *Washingtonia* sp. palm, bar = 20 nm. (TUM) Pleomorphic bodies with tri-unit membrane. (RLG) Ribosome-like granules. (DLF) DNA-like fibrils.

phytoplasma cells collected from bands of the density gradient are shown in Fig. 3. Numerous pleomorphic bodies with small vesicles resembling budding structures of phytoplasma were observed. The symmetrical trilaminar membranes can also be clearly seen. We observed that the central fibril of DNA-like material in the cells was surrounded by a granular peripheral zone that contained the ribosome-like granules. No phytoplasma-like body was observed in the purified suspension from all 12 healthy tested palms.

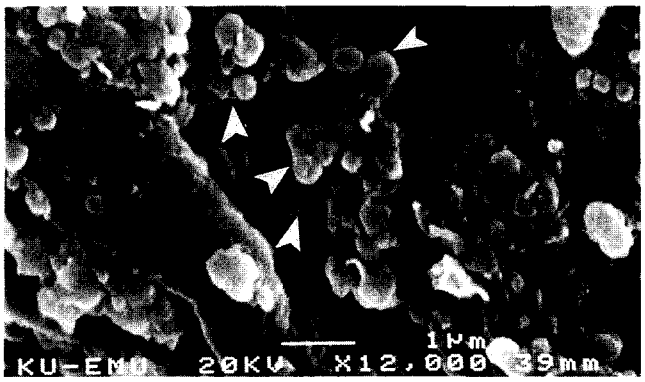

Fig. 4. SEM micrograph of freeze fractured leaf tissue showing various morphology of phytoplasma cells in spherical forms, branching forms appearing slightly flattened when lying against the plasma membrane, also showing some forms of possible binary fission, bar = 1 μm.

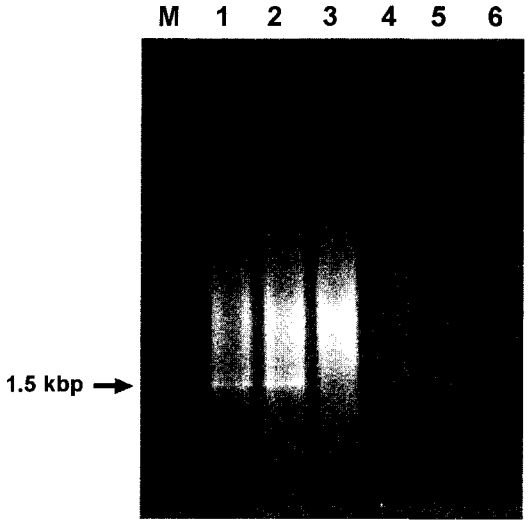

Fig. 5. Direct polymerase chain reaction (PCR), amplification of phytoplasmal 16S rDNA sequences from naturally infected *Washingtonia* sp. Lane M: Lambda DNA digested with *Hind* III; Lane (1): positive control phytoplasma from diseased wind mill palm; Lanes (2): diseased palms; Lanes (3 & 4): healthy *Washingtonia* sp. palms from different locations.

Freeze Fracture SEM. The phytoplasma was confined to the phloem tissues and sieve tubes (Fig. 4). Magnification of the phloem tissues revealed varying concentrations of phytoplasma cells in the sieve tubes and disclosed the effects of the infection agents on the host plant. Phytoplasma was observed in the shapes of spherical bodies with

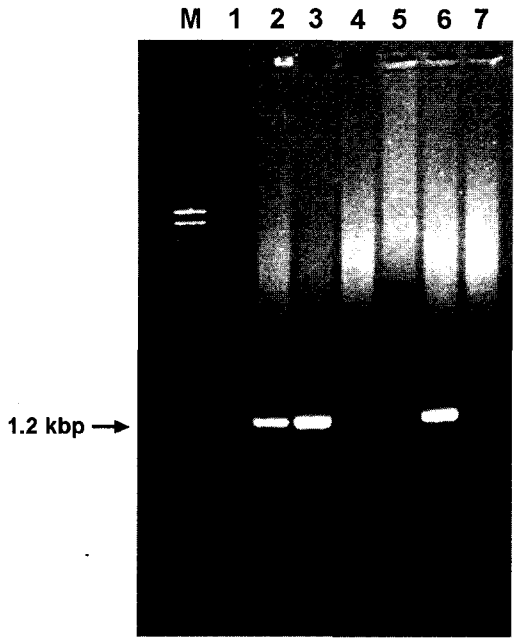

Fig. 6. Nested amplification of phytoplasma 16S rDNA sequences using nested primer pairs (R16F2n/R2) of naturally infected *Washingtonia* sp. PCR products (1.2 kbp) were analysed on a 1% agarose gel. Lane (M): ladder; Lanes (1 & 7): negative control, water; lane (2): positive control from Florida, USA; Lanes (3 & 6): infected *Washingtonia* sp. palms from Wafra; and Lanes (4 & 5): Healthy palms from Wafra.

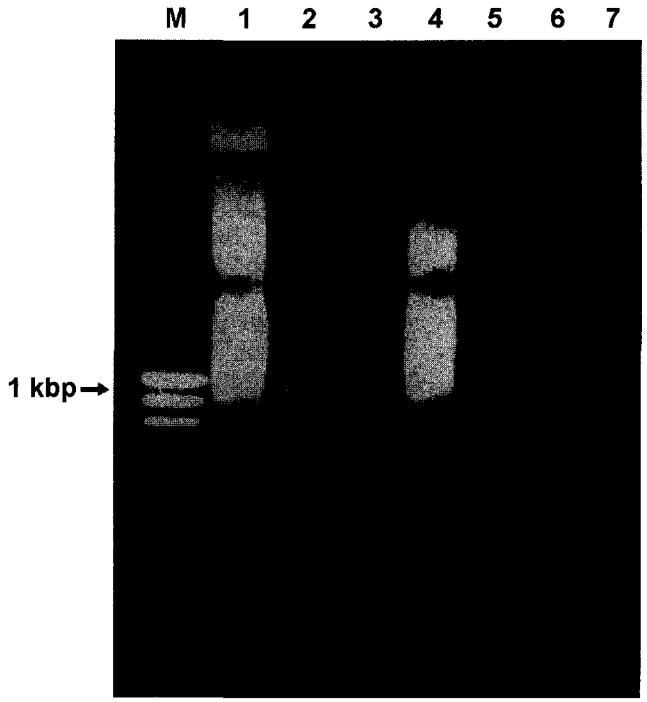

Fig. 7. Pathogen-specific PCR for detection of palm LY phytoplasma in ornamental palms *Washingtonia* sp. and date palms *Phoenix dactylifera* L. in Kuwait. PCR products (1.0 kbp) were analysed on a 1% agarose gel following 35 cycles of amplification. Lane (M): Ø 174 RFI DNA *Hae*III digest fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, & 72; Lane (1): Healthy *Phoenix dactylifera* L; Lanes (2 & 5): infected *Phoenix dactylifera* L; Lane (3): Healthy *Washingtonia* sp.; and Lanes (4, 6 & 7): infected *Washingtonia* sp.

buds, branching forms, and shapes which are presumably cells in the process of fission. No structures similar to these shapes were found in phloem cells of healthy plants.

Direct PCR amplification of 16S rDNA gene. Using universal primer set of R16mF2/R16mR1, DNA fragments of about 1.5 kbp were directly amplified from position 53 to 1487 on the 16S rDNA gene sequence extracted from infected tissues. This band matched with that of the phytoplasma positive control sample of diseased windmill palm. No amplified-DNA bands were observed in DNA samples that were extracted from healthy test palms (Fig. 5).

Nested-PCR for 16S rDNA Gene Amplification. Nested primer pair R16F2n/R16R2 resulted in the amplification of 16S rDNA fragments of about 1.2 kbp representing the position 152 to 1397 on the gene sequence (Fig. 6). Healthy appearing palms produced no DNA amplified PCR products.

A DNA product of about 1 kbp was specifically amplified by PCR in reaction mixtures containing template DNA derived from heart tissues of phytoplasma-affected date palms *P. dactylifera* using specific primer pairs LY1 and LY2 designed for lethal yellowing phytoplasma-specific genomic DNA isolated from a diseased windmill palm (*Trachycarpus fortunei*). No DNA products were amplified by PCR from similar mixtures containing template DNA extracted from healthy test samples (Fig. 7).

RFLP Analysis. PCR amplified rDNA products of about 1.2 kbp were compared by restriction fragment length polymorphism (RFLP) typing with *Taq*I restriction endonuclease enzyme. Using PAGE separation of the digested amplified rDNA products with *Taq*I endonucleases, DNA fragments of about 355 and 861 bp were obtained (data not shown).

Discussion

The objective of this study was to identify the presumptive causal agent of a yellowing disease in ornamental palms *Washingtonia* sp. grown in Kuwait. Following a random sampling of affected ornamental palms from different geographical locations in Kuwait, it was obvious that a yellowing disease was rapidly spreading in all sites visited. The symptomatology revealed similar patterns of infection leading to a conclusion that the plants were affected by yellows disease. Yellows symptom alone is insufficient evidence of phytoplasma etiology. The devastating effect of

yellowing disease on palms has attracted global concern.

In fluorescence microscopy, the phytoplasmal DNA appeared as a blue-white fluorescent specks or aggregates in sieve elements of the infected phloem tissues, whereas healthy sieve tubes remained typically dark. Preliminary examination by fluorescence microscopy of transverse sections of the leaves revealed only occasional accumulation of stains in a few phloem sieve tubes. The staining results suggest a low titer of phytoplasma in the affected palms. However, in case of severe infection, the intensity of fluorescence increased as the disease progressed. The pattern observed according to our results is in agreement with Hiruki and Chen (1981).

Many pleomorphic and spherical forms of phytoplasma with budding appearance were observed in electron microscopy according to Hirumi and Maramorosch (1969). This allowed for a hypothesis that phytoplasmas reproduce by budding and/or binary fission as with other Mollicutes. Binary fission was observed in several cases in our study. The phytoplasma cells also showed a well-defined trilaminar unit. Generally, the various morphotypes of phytoplasma cells seen in the purified preparations were similar to those found *in situ* in diseased plants. The size range of spherical phytoplasma observed in the phloem cells was consistent to results reported by others (Maramorosch and Phillips, 1981, Waters and Hunt, 1980).

The ultimate, proof of phytoplasma association can be obtained by several molecular techniques. The availability of highly sensitive molecular biology technique, such as nested PCR with the use of specific primers for different phytoplasma phylogenetic groups (Lee et al., 1994), made it possible to detect and identify phytoplasma in declining palms. The 16S rDNA was amplified by using a universal primer pair R16mF2/R1 followed by nested PCR with primer pair R16F2n/R2 which amplifies from position 152 to 1397 on the phytoplasmal 16S rDNA gene sequence according to Lim and Sears, 1989.

The disease causal-organism can be identified by using this initially amplified DNA segment as a template for further PCRs (*i.e.* nested PCR) using primers that only amplify DNA from that particular phytoplasmas. The use of nested PCR enabled the detection of a secondary phytoplasma or cryptic phytoplasma in a given specimen in case of mixed infection Lee et al. (1994). In the absence of such evidence for mixed infection in the study, it is safe to conclude that yellowing is caused by a single phytoplasma (Bertaccini et al., 1995). Nested-PCR assay proved to be more specific than universal PCR, and permitted detection of phytoplasmas in some palms before the onset of noticeable symptoms. Some DNA samples tested negative by universal PCR and then positive by nested PCR which may be an indication of the presence of contamination with other components at concentrations inhibitory to PCR. The present study proved that detection of the yellowing phytoplasma was consistent and reproducible for the affected palm species examined when DNA templates DNAs for PCR were derived either from young, unemerged influorescence or from heart tissues.

A DNA product of about 1 kbp was specifically amplified by PCR in reaction mixtures containing DNA templates derived from infected heart tissues with phytoplasma using specific primer pairs LY1 and LY2 designed for lethal yellowing phytoplasma-specific genomic DNA isolated from a diseased windmill palm *Trachycarpus fortunei.*

For further characterization, the nested PCR product can be subjected to restriction fragment length polymorphism (RFLP). DNA fragments amplified in nested PCR with primer pair R16F2/R2 were compared by RFLP analysis. The RFLP analysis of infected *Phoenix dactylifera* L. in Kuwait (Al-Awadhi et al., 2001, Kuwait University, unpublished data) confirmed identity with coconut lethal yellowing phytoplasma strains belonging to 16SrIV-A group (Gundersen and Lee, 1996, Lee et al., 1998, Lee et al., 1993). This provides a simple, reliable and rapid means for identification of the phytoplasma causing problems of palms in Kuwait.

References

Bertaccini, A., Vibio, M. and Stefani, E. 1995. Detection and molecular characterization of phytoplasmas infecting grapevine in Liguria (Italy). *Phytopathol. Medit.* 34:137-141.

Davis, M. J., Kramer, J. B., Ferwerda, F. H. and Brunner, B. R. 1996. Association of bacterium and not a phytoplasma with papaya bunchy top disease. *Phytopathology* 86:102-109.

Doi, Y., Teranaka, M., Yora, K. and Asuyama, H. 1967. Mycoplasma or PLT group like microorganisms found in phloem elements of plants infected with mulberry dwarf, potato witches brooms, aster yellows, or paulonia witches broom. *Ann. Phytopathol. Soc. Jap.* 33:259-266.

Doyle, J. J. and Doyle, J. L. 1990. Isolation of plants DNA from fresh tissue. *Focus* 12:13-15.

Gundersen, D. E. and Lee, I.-M. 1996. Ultrasensitive detection of phytoplasma by nested-PCR assays using two universal primer pairs. *Phytopathol. Medit.* 35:114-151.

Gundersen, D. E., Lee, I. -M., Rehner, S. A., Davis, S. A. and Kingsbury, D. T. 1994. Phylogeny of mycoplasmalike organisms (phytoplasmas): A Basis for their classification. *J. Bacteriol.* 176:5244-5254.

Hiruki, C. and Chen, M. H. 1981. Fluorescence microscopy in diagnosis of tree diseases associated with mycoplasmalike organisms (MLO). Proc. XVII IUFRO World Congress. Dir. 2:317-322.

Hirumi, H. and Maramorosch, K. 1969. Further evidence for a mycoplasma etiology of aster yellows. *Phytopathology* 59: 1030-1031.

Humphreys, W. J., Spurlock, B. O. and Johnson, J. S. 1974. Critical point drying of ethanol-infiltrated cryofractured specimens for scanning electron microscopy. pp. 275-281. In: Proc. 7th *Illinois Institute Technology Scanning Electron Microscopy.*

Krieg, N. R. and Holt, J. G., eds., 1984. *Bergey's Manual of Systemic Bacteriol.* Vol. 1. Williams and Wilkins, Baltimore MD, USA.

Lee, I. -M., Gundersen, D. E., Hammond, R. W. and Davis, R. E. 1994. Use of mycoplasmalike organism (MLO) group specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology* 84:559-565.

Lee, I. -M., Gundersen-Rindal, D. E., Davis, R. E. and Bartoszyk, M. 1998. Revised classification scheme of phytoplasma based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Int. J. Syst. Bacteriol.* 48:1153-1169.

Lee, I.-M., Hammond, R. W., Davis, R. E. and Gundersen, D. E. 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma like organisms. *Phytopathology* 83: 834-842.

Lim, P. and Sears, B. B. 1989. 16S RNA sequences indicates that plant pathogenic mycoplasma-like organisms are evolutionarily distinct from animal mycoplasmas. *J. Bacteriol.* 171: 5906.

Maramorosch, K. and Phillips, D. M. 1981. Scanning electron microscopy of aster yellows and cactus spiroplasmas. *Phytopathol. Z* 102:195-200.

Montasser, M. S., Al-Sherada, A., Ali, N. Y., Nakhla, M. K., Fraj, P. P. and Maxwell, P. 1999. A single DNA of tomato yellow leaf curl geminivirus causing epidemics in the State of Kuwait. *Kuwait J. Sci. Engineering* 26:127-142.

Nienhaus, F., Schuiling, M., Gliem, G. and Schinnzer, U. 1982. Investigations on the etiology of the lethal disease of coconut palm in Tanzania-Z. *Pflkrankh. Pfischutz.* 89:185-193.

Sears, B. B. and Kirkpatrick, B. C. 1994. Unveiling the evolutionary relationship of plant pathogenic mycoplasma like organisms. *ASM News* 60:307-312.

Seemuller, E., Schneider, B., Murer, R., Ahrens, U., Daire, X., Kison, H., Lorenz, K. -H., Firrao, G., Avinent, L., Sears, B. B. and Stackebrandt, E. 1994. Phylogenetic classification of phytopathogenic Mollicutes by sequence analysis of 16S ribosomal DNA. *Int. J. Syst. Bacteriol.* 44:440-446.

Sinha, R. C. 1974. Purification of mycoplasma-like organisms from China aster plants affected with clover phyllody. *Phytopathology* 64:1156-1158

Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electronmicroscopy. *J. Ultrastruc. Res.* 26:31-43.

Thomas, D. L. 1979. Mycoplasma-like bodies associated with lethal declines of palms in Florida. *Phytopathology* 69:928-934.

Waters, H. and Hunt, P. 1980. The in vivo threedimensional form of a plant mycoplasma like organisms by the analysis of serial ultra thin sections. *J. Gen. Microbiol.* 116:111-131.