J. Plant Biol., Vol. 28 (1), April, 2001, pp. 55–60.
© Society for Plant Physiology and Biochemistry

ARTICLE

Biochemical Evidences for Purification of Phytoplasma of Root (wilt) Disease of Coconut

M. Mayilvaganan*, Alka Gupta and K. R. Chithra
Central Plantation Crops Research Institute, Regional Station, Kayangulam, Krishnapuram 690 533, India

MS received 29 June 2000; accepted 29 November 2000

Phytoplasma of root (wilt) disease of coconut *(Cocos nucifera* L.) has been purified from tender leaf tissues of diseased palms by Percoll-discontinuous density gradient centrifugation (PDDGC). Purified phytoplasma fractions were checked by biochemical methods such as ultraviolet-absorption spectrum (190–310 nm) and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). PDDGC produced a single turbid zone of phytoplasma at the top of 30% step gradient from diseased leaf samples, which was not seen in samples from healthy leaf. Phytoplasma from this fraction had an absorption maximum near 200 nm. On further fractionation, the protein and nucleic acid components of phytoplasma showed absorption peaks at 280 and 260 nm respectively. The protein of phytoplasma from diseased leaf samples produced three major peptides of 29, 28 and 18.5 kDa in SDS–PAGE.

Keywords: Coconut, root (wilt) disease, phytoplasma, purification.

Introduction

Root (wilt) disease (RWD) of coconut, an economically important disease of this plantation crop, is caused by phytoplasma. Consistent presence of phytoplasma in tissues of roots, tender rachilla, petioles and developing leaf bases of diseased coconut and its absence in healthy palms has been established by electron microscopy (Solomon *et al.*, 1983a). The evidences for phytoplasma as the causal agent of RWD are also based on therapeutic effect of oxytetracycline-HCl antibiotic in inducing remission of symptoms in significant number of palm trials, but not by penicillin (Pillai *et al.*, 1991), transmission of the disease from diseased palms to healthy coconut seedlings through insect vector *Stephanitis typica* (Distant) (Mathen *et al.*, 1987) and from coconut to periwinkle (*Catharanthus roseus* L.), the phytoplasma indicator host through dodder laurel (*Cassytha filiformis* L.) (Sasikala *et al.*, 1988).

Phytoplasmas are non-culturable, cell wall-less, prokaryotic pathogens which have been reported to cause more than 600 plant diseases (McCoy *et al.*, 1989). Like many other phytoplasmas, the root (wilt) phytoplasma (RWP) has remained non-culturable *in vitro*. Since attempts to isolate and cultivate RWP were unsuccessful, extraction of this pathogen from tissues of coconut palm and its purification for biochemical characterisation and to produce RWP-specific antiserum for its detection are necessary. Recently, molecular techniques have been developed to amplify the 16S rDNA of phytoplasma using universal and specific oligoprimers (Ahrens and Seemuller, 1992; Gomez *et al.*, 1996; Griffiths *et al.*, 2000).

Jiang and Chen (1987) have developed a method to purify aster yellows' phytoplasma to relatively higher concentration by Percoll-step gradient. Monoclonal antibodies have also been employed for purification using immunoaffinity technique (Jiang *et al.*, 1988; Seddas *et al.*, 1995).

This paper reports the purification of RWP by PDDGC from leaf tissues of diseased coconut palms and assessing the purified phytoplasma cells by biochemical methods as a first step towards production of phytoplasma-specific antiserum.

Materials and methods

Source of plant materials

For diseased plant materials, coconut palms in the middle stage of RWD were selected and whitish non-

*For correspondence. (e-mail: cpcrikgm@md4.vsnl.net.in)

chlorophyllous spear leaf were collected and used for purification of phytoplasma. A total of 18 diseased palms were sampled during 1998–1999 for the study. Similar type of leaf tissues was also collected from healthy palms from Tirunelveli district of Tamil Nadu, which is a RWD-free tract. The sample leaf tissues were subjected to serological testing with RWD-specific antiserum before used for purification (Solomon *et al.*, 1983b).

Processing of materials

The method adopted was essentially of Jiang and Chen (1987) who devised and employed it for purification of phytoplasma of aster yellows' disease from lettuce plant. The protocol was modified to suit to the fibrous and phenol-rich coconut leaf tissues. Soon after collecting, the leaf samples were thoroughly washed in distilled water and the moisture was completely removed. A 100 g of tissue was chopped into small pieces, chilled at 4°C for 30 min and ground in 300 ml chilled isolation medium containing 0.3 M D-mannitol, 4 mM L-cysteine, 30 mM (N-morpholino)-propane sulfonic acid (MOPS) buffer, 1 mM ethylenediamine tetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP), pH 7.2 for 30 s with intervals for a total of 2 min. The ground material was filtered through three layers of muslin cloth and the remaining fibrous material was ground in 200 ml of isolation medium twice for 30 s. The sap was squeezed through muslin cloth and the pooled total sap was centrifuged at 1,500 *g* for 8 min. The supernatant was centrifuged at 35,000 *g* for 30 min in Beckman-L5-50B ultracentrifuge using fixed angle rotor type 50.2Ti. The pellets were dissolved in 60 ml chilled suspending medium containing 0.3 M D-mannitol and 20 mM MOPS buffer, pH 7.0. The suspension was again subjected to low- and high-speed differential centrifugation. The final pellets were suspended in about 10 ml of suspending medium and clarified at 1,500 *g* for 8 min. Similarly, leaf samples collected from healthy palms were also treated by the same protocol as soon as it was brought to laboratory.

Preparation of discontinuous density gradients and centrifugation

The density medium (nine parts of Percoll, from Pharmacia Biotech, Sweden and one part (v/v) of 2.5 M sucrose) and diluting medium (0.25 M sucrose and 10 mM MOPS buffer, pH 7.0) were used for forming a four step-density gradients of 15% (9 ml), 30% (9 ml), 50% (5 ml) and 60% (5 ml). The step gradients prepared in 25 × 89 mm ultra-clear 38.5 ml quick seal G. max centrifuge tubes (Beckman Instrument Inc., Palo Alto, CA) were over-layered with about 0.5 to 0.8 ml of partially purified phytoplasma preparation. The centrifugation was carried out at 20,000 *g* for 20 min once and again for 10 min. After centrifugation, the zone showing turbidity in diseased leaf sample loaded tube was collected and diluted with suspending medium. Fraction from the corresponding zone from healthy material loaded tube was also collected and diluted with suspending medium.

The fractions were centrifuged at 1,00,000 *g* for 120 min to remove Percoll and the pellets enriched with phytoplasma were collected in about one ml of phosphate buffered saline (PBS) pH 7.0. This was dialysed overnight against PBS and finally clarified by centrifuging at 1,500 *g*.

UV absorption spectrum

One ml of purified phytoplasma in PBS was diluted to two ml. The UV-absorption spectrum of the diluted sample was recorded in a spectrophotometer (ATI UNICAM with UV/visible vision software V3.00) between 190 and 310 nm.

Fractionating into protein and nucleic acid

The fractionation was carried out using the methods described by Ralph and Bergquist (1967) for viruses with modifications. About 8 ml of undiluted preparation was mixed with an equal volume of water-saturated phenol (100 g phenol with 40 ml distilled water and 0.14 g 8-hydroxyquinoline) and mixed for 15 min in a semi-clear tube. The mixture was centrifuged at 12,000 *g* for 10 min to clear the emulsion into lower phenolic phase and upper aqueous phase. The upper phase was removed and re-extracted twice with three ml of phenol. The final aqueous phase was mixed with two thirds volume of ice-cold isopropanol and stored at 4°C overnight to precipitate the nucleic acid. The nucleic acid was pelleted by centrifuging at 15,000 *g* for 20 min. The phenol resi-

dues were removed by washing with ice-cold 70% ethanol containing sodium acetate. The pellets were air dried and dissolved in sterile double distilled water. To the total phenolic phase, 20 ml of methanol and several small crystals of sodium acetate were added and the resulting protein precipitate was collected by centrifuging at 12,000 *g* for 10 min. The pellets were washed thrice with methanol and dissolved in sterile distilled water by heating to 60°C. The UV-absorption spectra of the protein and nucleic acid samples were recorded.

SDS–PAGE of protein

Protein fractionated from purified phytoplasma was concentrated and processed for SDS–PAGE according to Laemmli (1970). The protein was estimated by Lowry's method (Lowry *et al.*, 1951) and adjusted to the concentration of 0.2 mg per millilitre in water and mixed with solublisation sample buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 2% β–mercaptoethanol and 1% bromophenol blue) 1 : 4 v/v and heated at 98°C for 4 min. Protein samples containing 0.5 µg of protein were resolved on a discontinuous gradient gel with 4% acrylamide in the stacking gel and 12% in the resolving gel, in a Bio-Rad protein dual slab cell (Richmond, CA). Samples were run at 40 mA till the tracking dye reached the separating gel, after which the gel was run at 60 mA for 120 min. The electrophoresed gels were stained in 0.1% CBB R-250 overnight and destained.

Results

Purification by PDDGC

Centrifugation at 20,000 *g* for 20 min produced a single turbid zone at the top of 30% step gradient in the tube containing diseased coconut samples. This turbid zone was totally absent in the healthy sample tube. The turbid zone was not observed other than 30% step gradient and also in healthy sample (Figure 1). After centrifugation of Percoll-gradient for 20 min, the turbid zone was more than 10 mm wide. However, the second consecutive centrifugation was resorted to for getting narrow and concentrated zone of about 5–10 mm. The turbid band produced by the

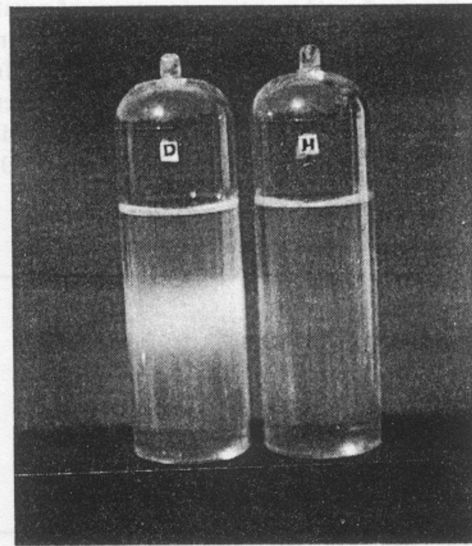

Figure 1. Discontinuous density gradients (15, 30, 50 and 60%) after two consecutive centrifugation at 20,000 *g* for 30 min, D, Diseased preparation showing turbid zone at the top of 30% step gradient; H, Healthy preparation.

infected plant material was opalescent, thick and brown in colour. When more than one ml of partially purified phytoplasma was overlayered, part of the preparation remained on top of gradients or a wide zone of band was produced spreading through the entire 15% step gradient. Hence, only 0.5 to 0.8 ml of partially purified phytoplasma preparation was used for overlayering the Percoll-gradient. When 0.5 ml of partially purified phytoplasma preparation obtained from 25 *g* infected tissue was loaded on the Percoll-gradient, it produced a thin and less concentrated turbid band in the 30% step gradient. Therefore we used 100 g tissue for purification to obtain more concentrated band of phytoplasma.

The same results were obtained when 2,000 *g* of leaf samples (100 g each time) from 18 diseased palms and samples from 10 healthy palms were processed for purification.

UV-spectrum

The differences in absorption spectrum of phytoplasma purified from infected material and corresponding preparation from healthy plant material are

shown in Figure 2a. Absorption spectrum of purified phytoplasma showed absorption maximum near 200 nm and the peak was sharper. Fractions from other zone in diseased sample tube and a fraction from the corresponding zone (30%) of the gradient of healthy sample tube showed no peak near 200 nm or at any other wavelength of the spectrum. When the purified preparations obtained from 25 g tissue were analysed, the difference in peak of absorption at 200 nm between healthy and diseased preparations was greatly reduced.

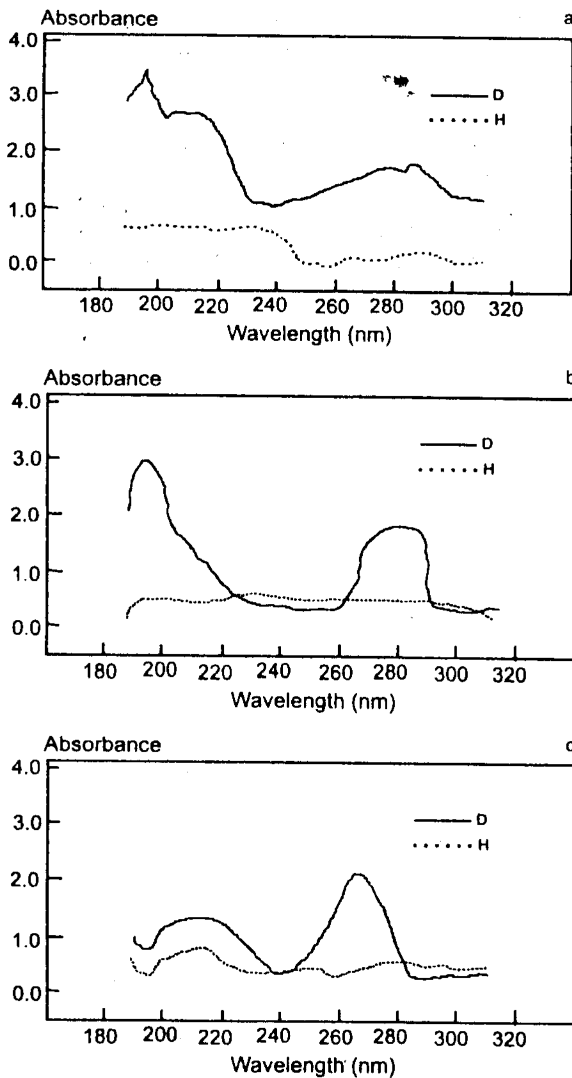

Figure 2. a, Absorbance (190–310 nm) of fractions from diseased and healthy preparations. D, purified phytoplasma fraction from diseased preparation and H, corresponding fraction from healthy preparation. b, Absorbance of protein of purified phytoplasma fraction from diseased sample (D) and corresponding fraction from healthy sample (H). c, Absorbance of nucleic acid of purified phytoplasma fraction from diseased sample (D) and corresponding fraction from healthy sample (H).

When purified phytoplasma was fractionated into their major cellular components of protein and nucleic acid and subjected to UV-absorption profile from 190 to 310 nm, the solution containing protein produced two peaks at 200 and 280 nm (Figure 2b) and solution of nucleic acid at 260 nm (Figure 2c). No peaks were produced in fractions from healthy sample. The absorption peak at 200 nm for protein was not sharper (Figure 2b) as observed in purified phytoplasma preparation (Figure 2a).

SDS–PAGE of protein

The SDS-PAGE of protein fractionated from RWP purified from diseased palm by PDDGC is shown in Figure 3 along with fraction from healthy palm sample. Peptide profiles of purified phytoplasma from diseased palm (lane 1) showed three bands of 29, 28 and 18.5 kDa. No band was observed in healthy sample fraction (lane 3). Among the three peptides observed from diseased plant material, the peptide band of 18.5 kDa was more prominent than other two peptides, 29 and 28 kDa (Figure 3, lane 1). The protein profile results obtained in SDS–PAGE were consistent and reproducible.

Discussion

Phytoplasma inhabits in sieve tubes of phloem tissues and their amount is more in tissues that contain greatest concentration of functional sieve tubes. In coconut palms, heart tissues followed by just emerging and growing leaf bases were found to possess more amounts of sieve elements and in turn more phytoplasma. In our studies, whitish spear leaf emerging from heart tissues were used. The idea was to collect best materials from palm without destroying it. The mature tissues of coconut contained only a fewer degenerated phytoplasma without internal ribosomes and DNA fibrils (Solomon *et al.*, 1987).

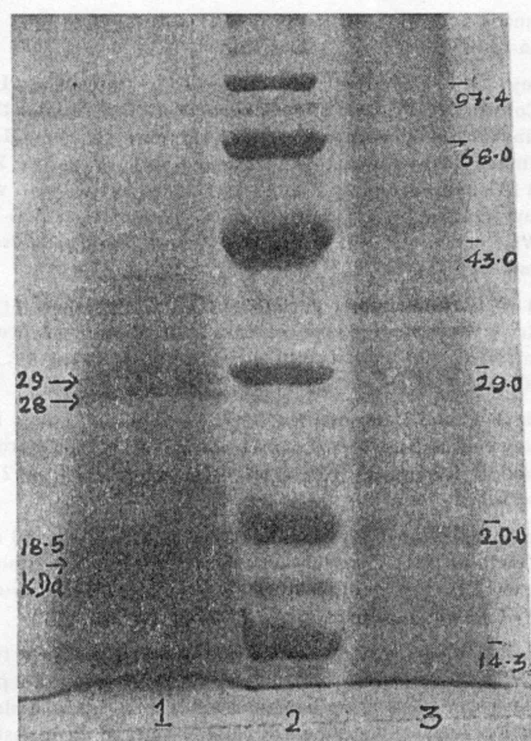

Figure 3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis profiles of proteins (samples containing 0.5 µg of proteins); lane 1: Purified phytoplasma from diseased sample – the arrows point to three protein bands (29, 28 and 18.5 kDa); lane 2: Molecular masses of standard proteins and lane 3: preparation from healthy sample.

The RWD could be detected by Ouchterlony test (Solomon *et al.*, 1983b) using root (wilt) antiserum. Using this test, the severity of the disease can also be determined based on the intensity of the reaction with precipitin line being pronounced more in the early stage of disease.

Jiang and Chen (1987) and Gomez *et al.* (1996) have used this method to purify aster yellows and china-tree decline phytoplasmas respectively. The properties and functions of various components of isolation and suspending media and of Percoll and Percoll gradients method have been well documented by Jiang and Chen (1987). In the present study, the duration of grinding and proportionate quantity of isolation medium to leaf tissue was increased to ensure complete disruption of fibrous coconut leaf material. The PVP content in isolation medium was also increased to 1% to help removal of phenolics from tissue. The duration of the entire purification schedule was kept minimum to prevent considerable degeneration of phytoplasma as they (aster yellows phytoplasmas) have maximum survival period of 72 h *in vitro* (Smith *et al.*, 1981).

After scrutinising the various combinations of step gradients used by Jiang and Chen (1987), the four step gradient of 15, 30, 50 and 60% was employed in this study so that the density of the RWP fall within densities of these gradients. When the step gradients were centrifuged at 20,000 *g* for 20 min, the turbidity zone was wider and the best result was obtained when centrifuged for another 10 min. In the present study, the turbid band produced by infected leaf tissue was wide and thick (Figure 1). This could be due to the amount of tissue (100 g) used for purification and also presence of high level of phenolics in coconut leaf material. Seddas *et al.* (1995) and Gomez *et al.* (1996) previously used only 12 g and 30 g tissues respectively to purify flavescence doree and china-tree decline phytoplasmas and they obtained a thin turbid band.

Formation of single turbid zone only in diseased sample tube but not in healthy material proves that the zone is an indication of accumulation of phytoplasma. Observation of a single absorbance peak (near 200 nm) from the fraction of turbid zone of diseased sample and its marked difference of absorbance with that of healthy sample, and observation of characteristic peaks at 280 and 260 nm for protein and nucleic acid from diseased samples showed that the turbid zone had cell-like bodies with protein and nucleic acids, the predominant components of cells. Aster yellows' phytoplasma were shown to contain a trilaminar unit membrane, cytoplasmic ribosome granules and a nuclear area with DNA fibrils (Jiang and Chen, 1987).

Detection of protein bands (29, 28 and 18.5 kDa) only from diseased sample proves that the cell-like bodies were indeed RWP. Jiang *et al.* (1988) have also reported a major protein of 18.5 kDa and two minor proteins from aster yellows phytoplasma whereas Seddas *et al.* (1995) have reported two major proteins of 55 and 19 kDa and a few minor proteins from flavescence doree phytoplasma.

Phytoplasmas are now detected by PCR amplification of 16S rDNA using universal and phytoplasma-specific primers as of late done in sandal spike and mulberry dwarf phytoplasmas (Thomas and Balasundaran, 1999; Kawakita *et al.*, 2000). However, purification of phytoplasma and production of antiserum/antibodies specific to RWP for its detection in large number of samples will be a valuable tool.

References

Ahrens U and Seemuller N 1992 Detection of DNA of plant pathogenic mycoplasma-like organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene; *Phytopathology* **182** : 828–832.

Gomez G G, Conci L R, Ducasse D A and Nome S F 1996 Purification of phytoplasma associated with China-tree (*Melia azedarach* L.) decline and the production of a polyclonal antiserum for its detection; *J Phytopathol* **144** : 473–477.

Griffiths H M, Harrison N A and Sinclair W A 2000 Phytoplasmas identified in Virginia creeper represent a new subgroup within the 16S rRNA group V (Abst); *Phytopathology* **90** (supp) : S30.

Jiang Y P and Chen T A 1987 Purification of mycoplasma-like organisms from lettuce with aster yellows disease; *Phytopathology* **77** : 749–953.

Jiang Y P, Lei J P and Chen T A 1988 Purification of aster yellow agent from diseased lettuce using affinity chromatography; *Phytopathology* **78** : 828–831.

Kawakita H, Saiki T, Wei W, Mitsuhashi W, Watanabe K and Sato M 2000 Identification of mulberry dwarf phytoplasmas in the genital organs and eggs of leaf hopper *Hishimonoides sellatiformis*; *Phytopathology* **90** : 909–914.

Laemmli U K 1970 Cleavage of the structural proteins during assemply of the head of bacteriophage T4; *Nature* **227** : 680–685.

Lowry O H, Rosebrough N J, Farr A L and Randall R J 1951 Protein measurement with folin phenol reagent; *J Biol Chem* **193** : 265–275.

Mathen K, Solomon J J, Rajan P and Geetha L 1987 Electron microscopic evidence on the role of *Stephanitis typica* (Distant) as vector of coconut root (wilt) disease; *Curr Sci* **56** : 1339–1341.

McCoy R E, Caudwell A, Chang C J, Chiykowsky L N, Cousin M T, Dale J L, DeLeeuw G T N, Golino D A, Hacket K J, Kirkpatrick B C, Marwitz R, Petzold H, Sinha R C, Sugiura M, Whitcomb R F, Yang I L, Zhu B M and Seemuller E 1989 Plant diseases associated with mycoplasma-like organisms; in *The mycoplasmas* (eds) R F Whitcomb and J G Tully (New York, Academic Press) vol 5 pp 564–620.

Pillai N G, Chowdappa P, Solomon J J and Mathew J 1991 Remission of symptoms of root (wilt) disease of coconut injected with oxytetracycline-HCl; *J Plant Crops* **19** : 14–20.

Ralph R K and Bergquist P L 1967 Separation of viruses into components; in *Methods in virology* (eds) K Maramorosch and H Koprowski (New York, Academic Press) vol 2 pp 463–545.

Sasikala M, Mathen K, Govindankutty M P, Solomon J J and Geetha L 1988 Transmission of mycoplasma-like organisms from *Cocos nucifera* with root (wilt) to *Catharanthus roseus* by *Cassytha filiformis*; *Neth J Pl Pathol* **94** : 191–194.

Seddas A, Meignoz R, Kuszala C and Bouden-Padieu E 1995 Evidence for the physical integrity of flavescence doree phytoplasmas purified by immuno-affinity from infected plants or leaf hoppers and the plant pathogenicity of phytoplasmas from leafhoppers; *Pl Pathol* **49** : 971–978.

Smith A J, McCoy R E and Tsai J H 1981 Maintenance *in vitro* of the aster yellows mycoplasma-like organism; *Phytopathology* **71** : 819–822.

Solomon J J, Govindankutty M P and Mathen K 1987 Detection of mycoplasma-like organisms in root (wilt) disease affected coconut and its putative vector; in *Regional workshop on plant mycoplasma* New Delhi, 24–28 Feb.

Solomon J J, Govindankutty M P and Nienhaus F 1983a Association of mycoplasma-like organisms with coconut root (wilt) disease in India; *Z Pflkrankh Pflschutz* **90** : 295–297.

Solomon J J, Sasikala M and Shantha P 1983b A serological test for the detection of root (wilt) disease of coconut; in *Coconut research and development* (ed.) N M Nayar (New Delhi, Wiley Eastern) pp 401–405.

Thomas S and Balasundaran M 1999 Detection of sandal spike phytoplasma by polymerase chain reaction; *Curr Sci* **76** : 1574–1576.