Association of Two Ribonucleic Acid Species with Cadang-Cadang Disease of Coconut Palm

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ABSTRACT

Coconut palms with cadang-cadang, a disease of suspected viral etiology, contain two apparently disease-specific RNA species of low molecular weight. A survey of coconut palms, within and outside the area where the disease is known to occur, has shown that the two RNA species are found only in nucleic acid extracts of diseased palms, not in normal or stressed (but nondiseased) palms. They can be detected just before symptoms appear in developing young fronds, and are then retained as the leaves mature. They can also be detected at early, mid-, and late stages of disease development, and therefore may be useful in the diagnosis of the disease.

It is suggested that these RNA species may be components of the presumed cadang-cadang pathogen, and one of them, with a molecular weight of 84,000 daltons, a sedimentation coefficient of 7.5S, and a thermal denaturation curve with an approximate 10% hyperchromic shift at about 58 C, is similar to known members of the viroid group of pathogens. Failure to find conventional virus particles in preparations from which the disease-specific RNA's were obtained, and the sensitivity of the disease-specific RNA species to low concentrations of ribonuclease before deproteinization of preparations, supports the view that a viroid hypothesis for cadang-cadang disease should be seriously considered.

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Additional key words: yellow mottle decline, viroid hypothesis, diagnosis.

Cadang-cadang, or yellow mottle decline (2) of coconut (*Cocos nucifera* L.) was first reported to be a serious disease on San Miguel Island in the Philippines in 1931 (14), and it is now known to occur within an approximate 300-mile radius of its apparent origin. Considered to be the main threat to coconut production in the Philippines, and having caused an estimated loss of 12 million palms, it is listed as a disease of limited threat potential to world agriculture by Thurston(22). Despite considerable work, the causal agent and its mode of spread remain unknown (17). Symptomatology (13) and histological changes observed in diseased leaves (20) are not inconsistent with a viral etiology, but despite evidence for natural spread (18, 19), artificial transmission has not yet been convincingly demonstrated, nor have typical virus particles been observed in tissue. The recently observed 'virus-like particles' in chloroplasts of diseased palms (16) are almost certainly phytoferritin (7).

This paper reports the results of an attempt to further test the virus hypothesis for cadang-cadang disease by seeking unusual nucleic acid components in diseased palms.

MATERIALS AND METHODS.—*Survey.*—A survey of diseased and nondiseased palms was carried out, with samples being collected from one locality within the disease area, and two outside it (see Table 1). The nondiseased sample included palms stressed by such factors as waterlogging, "hard-pan", and nutrient deficiency, and showing some characteristics of the cadang-cadang disease such as chlorosis, stunting, and absence of inflorescences.

Leaflets were harvested from the middle third of fronds 5 or 15, counting downwards from the first opened frond in the crown of the palm. Fronds older than frond 3 and 4 on diseased palms show the characteristic leaf-spotting symptom.

Nucleic acid extraction and analysis.—Fifty grams of leaflet tissue was chopped by hand, blended for 1 minute with 200 ml of cold 0.1 M Na_2HPO_4 containing 0.1% sodium thioglycollate and 0.01 M sodium diethyldithiocarbamate, strained through muslin, and the filtrate clarified by centrifugation at 2,000 *g* for 15 minutes. To precipitate macromolecules and the putative "virus", solid polyethylene glycol (PEG 6000) was added (8, 23) to 5%, dissolved by stirring in the cold, and the precipitate was collected after 1-2 hours by centrifugation as above. Precipitates were resuspended in 2 ml 0.05 M sodium phosphate buffer (pH 7.2). Nucleic acids were extracted from this by adding 0.2 ml of 10% sodium dodecyl sulphate (SDS) and 1 ml of 90% aqueous phenol (with 0.1% 8-hydroxyquinoline), shaking for 1 hour at 25-30 C, centrifuging at 2,000 *g* for 10 min, collecting the aqueous phase, adding cold ethanol to 75%, and collecting the precipitate by centrifugation. After draining the precipitate, 0.3 ml of a preincubated solution of 0.1% *Streptomyces griseus* protease - 0.5% SDS - 0.1 M sodium acetate (12) was added, and the mixture was incubated at 37 C for 18 hours. A further phenol extraction was carried out, by adding 1 ml of sterile distilled water and 1 ml of the phenol solution, emulsifying the mixture for 25 minutes, and recovering nucleic acids by ethanol precipitation from the aqueous phase. Precipitates were drained, dissolved in 0.1 ml sterile electrophoresis buffer containing 1.0% SDS, and dialyzed against 0.5% SDS for 18 hours to remove a dialyzable component which affected electrophoretic mobility. Nucleic acids were again recovered by ethanol precipitation, and the dried precipitate was dissolved in

0.1 ml of sterile electrophoresis buffer containing 10% sucrose and bromophenol blue marker dye.

When nucleic acid preparations were to be transferred from the Philippines to the Waite Institute, the ethanol precipitate was washed with acetone, dried at low pressure, and a grain of dehydrated silica gel was added to the tube before sealing it with Parafilm. No change in the nucleic acid composition of stored samples was observed.

Polyacrylamide gels at 2.5% (with 0.5% agarose) (15) and 3.3%, and buffered with Tris-borate-EDTA (15), were used to separate nucleic acids. Gels were prerun at 2.0mA per gel for 1 hour at 4 C, 0.05 ml of the nucleic acid preparation was loaded, and electrophoresis was continued at 1.0mA per gel for 10-30 minutes, then at 2.0mA until the marker dye was within 15 mm of the bottom. The position of the dye was marked by injecting ink, and gels were stained in 0.01% toluidine blue in 5.0% acetic acid, then destained in water. To recover RNA, stained bands were excised, mixed with 1 ml of 10% SDS and 1.0 ml phenol, the gel was pulverized with one stroke of the pestle in a glass homogenizer, then shaken gently for 16 hours at 25 C. Another 1.0 ml of phenol was added, the mixture was centrifuged, and RNA was recovered from the supernatant by ethanol precipitation. RNA was dissolved in 1 ml of 0.01 SSC buffer (SSC buffer is 0.15 M

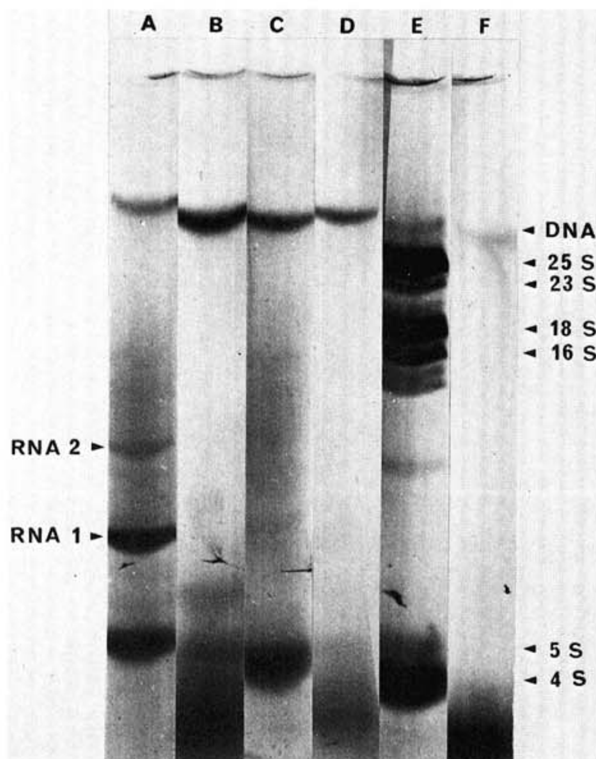

Fig. 1-(A to F). Separation of nucleic acid species, isolated from polyethylene glycol precipitates of diseased and normal palms, by electrophoresis on 2.5% polyacrylamide gels. **A)** Nucleic acids from diseased palms. **B)** (A) treated with 20 μg/ml ribonuclease (RNase) for 15 minutes at 20 C before loading. **C)** Nucleic acids from normal palm. **D)** (C) - RNase treated. **E)** Total nucleic acid extract from cucumber (marker). **F)** (E) - RNase treated.

sodium chloride, 0.015 M sodium citrate, pH 7.0), layered over a discontinuous cushion comprising 2.0 ml 40% sucrose and 2.0 ml 10% sucrose in 0.1 M Tris-HCl (pH 7.3), then overlaid with 6 ml of 0.01 SSC buffer. Tubes were centrifuged in a Spinco SW41 rotor at 38,000 rpm (180,000 *g*) for 16 hours at 2 C, fractions were collected from the bottom of the tube and RNA in the cushion fractions was collected by ethanol precipitation. Nucleic acids were also separated on 10-40% linear sucrose density gradients buffered in 0.1 M Tris-HCl (pH 7.3). Preparations were dissolved in 0.25 ml sterile distilled water, layered, then centrifuged for 16 hours at 38,000 rpm in a Spinco SW41 rotor. Ultraviolet (UV) absorbance profiles were obtained with an ISCO UV analyzer. RNA was recovered by ethanol precipitation. Thermal denaturation curves of RNA dissolved in 0.01 SSC buffer were obtained by heating at 0.5 C per min in a Unicam SP1800 ultraviolet spectrophotometer fitted with a temperature programme controller.

RESULTS.—*Association of two RNA species with diseased palms.*—Polyacrylamide gel electrophoretic separations of nucleic acids are shown in Fig. 1. Extracts from normal palms showed a slow-moving and a fast-moving band which stained relatively heavily (Fig. 1-C), and a few extracts showed several additional faint bands between these. The slow-moving band was ribonuclease (RNase)-resistant and was probably DNA; the fast band was RNase-sensitive (Fig. 1-D) and had the mobility expected for 5S RNA. In addition to these, extracts from infected palms showed two distinctive RNase-sensitive bands, RNA's 1 and 2 (Figure 1-A) which always occurred together, and had sedimentation coefficients estimated from electrophoretic mobility of about 7.5S and 11S. Their estimated molecular weights were approximately 8.4×10^4 and 2.04×10^5 respectively (Fig. 2). The relative amounts of RNA 1 and 2 varied, but RNA 1 was always in excess of RNA 2, and it stained heavily. It is estimated that the yield of these RNA's together was approximately 25 μg from 50 g of leaf.

The survey showed that RNA 1 and RNA 2 were detectable only in extracts of palms with typical symptoms of cadang-cadang (Table 1). Stressed nondiseased palms gave the same nucleic acid patterns as normal palms. RNA 1 and RNA 2 were readily detectable in palms at early, mid-, and late stages of disease (Table 1), and have also been detected in the young symptomless frond of a diseased palm 2 months before it was expected to show the typical leaf-spotting symptom.

Properties of disease-specific RNA species.—Electrophoretic mobility alone was insufficient to demonstrate the unrelatedness of RNA 1 and RNA 2 to normal host RNA's because, as mentioned above, faint bands with mobilities only slightly different from those of RNA 1 and RNA 2 were sometimes found in extracts from normal palms. Therefore, the thermal denaturation of RNA 1 and RNA 2 was investigated to see if they differed in other ways from normal palm nucleic acids. RNA 1 was recovered either from polyacrylamide gels or from sucrose density gradients. On sucrose density gradients, the nucleic acids isolated from diseased palms (Fig. 3-A) showed a small 5S peak, and a major peak (peak *a*) which, on the basis of comparison with marker transfer RNA and ribosomal

RNA's (10) isolated from cucumber, had an estimated sedimentation coefficient of 7.6S. The nucleic acids from normal palms showed a 5S peak (Fig. 3-B, peak *b*) and a 10S peak (Fig. 3-B, peak *c*). The 7.6S peak *a* therefore appears to be disease-specific, and was identified as RNA 1.

RNA 1 recovered directly from gels showed an uncharacteristically high UV absorption at 220 nm, and a minimum absorption at 238 nm, probably the result of a contaminant eluting with the RNA from the gel (1, 4). Sedimentation into a sucrose cushion removed this contaminant. The thermal denaturation curve of RNA 1 obtained this way showed a melting-point (Tm) at about 58 C, with a hyperchromic shift of about 10% (Fig. 4-A). RNA 1 recovered from sucrose density gradients also showed this thermal transition (Tm = 63 C), but it was less well defined because of a sloping baseline (Fig. 4-B), possibly resulting from the carry over of 5S RNA in the gradients. Peaks *b* (5S) and *c* (10S) from normal palms (Fig. 3-B), the latter probably comprising the material sometimes seen in polyacrylamide gels with a mobility similar to that of RNA 1, showed the hyperchromic shift (Fig. 4-C, D) expected for a single-stranded RNA (11). The 5S RNA's from both diseased and normal palms, and RNA 2 from diseased palms, when recovered from gels, showed no hyperchromic shift.

The form in which the cadang-cadang specific RNA 1 and RNA 2 exist in the PEG pellet was investigated in two ways. Firstly, the PEG 6000 concentrate of leaf sap was treated with ribonuclease (RNase) at 0.2 μg/ml for 10 hours at 0 C. This caused the loss of both RNA 1 and RNA 2 from subsequent nucleic acid extracts. Controls, where RNase was added after the incubation (that is, immediately before commencing the nucleic acid extraction) contained both RNA 1 and RNA 2, even when RNase was added at 2.0 μg/ml. RNA 1 and RNA 2, therefore, appear to be unprotected from RNase degradation before the deproteinization step, and may exist as free RNA in extracts. Secondly, PEG 6000 pellets were fixed in glutaraldehyde-osmium, embedded, and sectioned. No typical viruslike particles were observed in pellet sections from diseased palms, and no differences were observed between pellets obtained from normal and diseased palms–both contained large unstructured globular bodies of varying size, membrane-bound vesicles

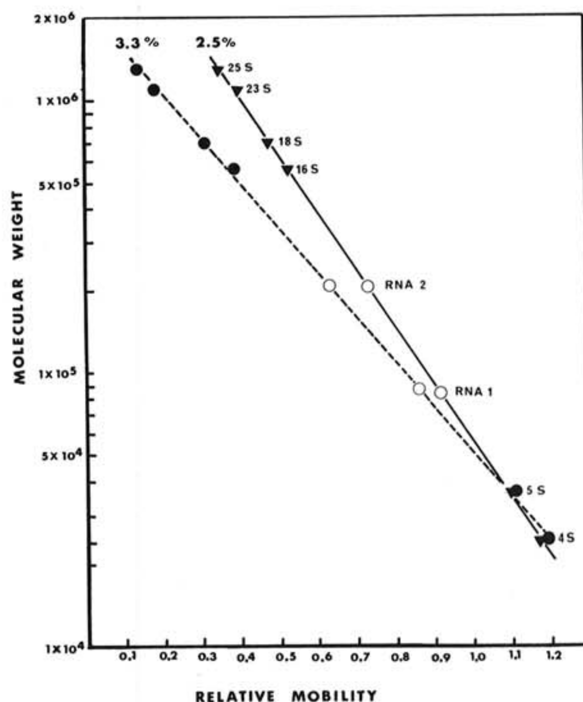

Fig. 2. Molecular weight estimation from mean electrophoretic mobilities of cadang-cadang specific RNA 1 and RNA 2 in 2.5% and 3.3% polyacrylamide gels, relative to that of bromophenol blue dye. Total cucumber nucleic acids, electrophoresed concurrently, were used as markers of molecular weight. In 2.5% and 3.3% gels, RNA 1 molecular weight was 8.2×10^4 and 8.5×10^4, respectively; and that of RNA 2 was 2.02×10^5 and 2.05×10^5.

which were either free or attached to the globules, and small electron-dense ferritin-like particles.

DISCUSSION.—Both RNA 1 and RNA 2 appear to be associated with cadang-cadang disease of coconut. Their appearance in fronds before symptoms appear, and their detection at early, as well as late, stages of disease development are consistent with their being associated with a presumed 'cadang-cadang' pathogen, rather than arising as a physiological response to infection. It is noteworthy that RNA 1 has properties remarkably

TABLE 1. Association of RNA 1 and RNA 2 with cadang-cadang disease in coconut palms

	Site of collection	Stage of disease	(Palms with RNA 1 and 2)/(Number in sample)
Normal palms	Philippines - Albay[a]	. . .	0/20
	- Davao	. . .	0/12
	Malaysia - Selangor	. . .	0/22
Stressed palms - No disease	Philippines - Albay[a]	. . .	0/6
	- Davao	. . .	0/12
Diseased palms	Philippines - Albay[a]	Early	4/5
		Mid	5/5
		Late	21/21

[a]Within known disease area. Cadang-cadang is not recognized in Davao or Malaysia.

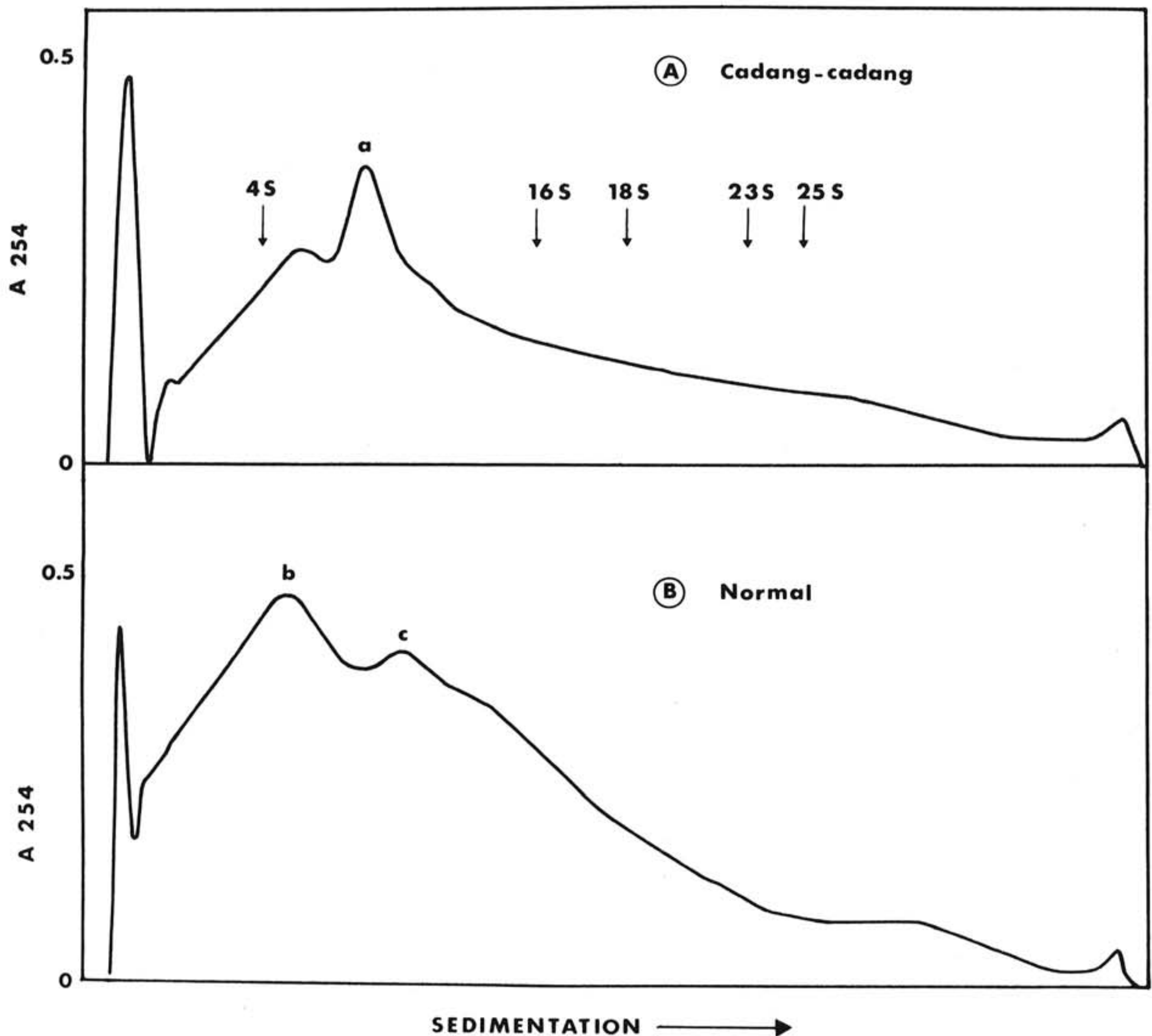

Fig. 3. Ultraviolet absorbance profiles of nucleic acids from diseased (A) and normal (B) palms separated in 10-40% linear sucrose gradients. Loading in B was double that of A. Reference sedimentation rates are provided by total cucumber nucleic acids, sedimented concurrently. Peak *a* is the disease-specific RNA 1. Peaks *b* and *c* are 5S and 10S components from normal palms.

similar to the potato spindle tuber viroid (PSTV). It has a molecular weight and sedimentation rate very close to that of PSTV (3, 6), and its thermal denaturation curve, which suggests some base paring, but which is atypical of either a double-stranded or a single-stranded RNA (11), again most closely resembles that of PSTV (4). Furthermore, the susceptibility of RNA 1 to RNase degradation before deproteinization of the PEG 6000 concentrate, and the inability to observe virus-like particles in PEG 6000 pellets suggests that the RNA 1 may be "naked" as is thought to be the case for viroids (3, 5, 9, 21). Although indirect, it appears that the evidence is now sufficiently strong for a "viroid" hypothesis for cadang-cadang to be seriously considered, but the final test of this will depend on the development of a method to test the infectivity of RNA 1 and RNA 2.

The polyacrylamide gel electrophoretic technique described here has potential as a diagnostic test for cadang-cadang. It is reliable, simple, and inexpensive, and is therefore well suited for use in the locality where the disease occurs. It should allow identification of disease at an early stage when visual identification is difficult.

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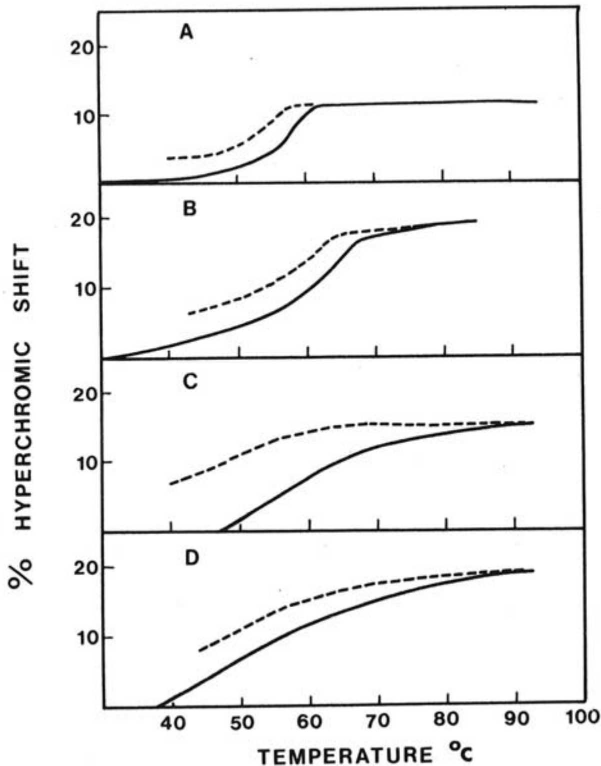

Fig. 4. Thermal denaturation (——) and cooling renaturation (- - -) curves in 0.01 SSC at 260 nm. **A**) RNA 1 recovered from polyacrylamide gels. **B**) RNA 1 recovered from sucrose density gradients (peak *a*, Fig. 3-A). **C**) 5S RNA recovered from normal palm on sucrose density gradients (peak *b*, Fig. 3-B). **D**) 10S RNA recovered from normal palm on sucrose density gradient (peak *c*, Fig. 3-B).

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