Tinangaja and Bristle Top, Coconut Diseases of Uncertain Etiology in Guam, and Their Relationship to Cadang-Cadang Disease of Coconut in the Philippines

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ABSTRACT

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The symptomatologies of tinangaja and bristle top, two disorders afflicting coconut palms on the island of Guam, were compared with cadang-cadang disease in the Philippines. Nucleic acids were extracted from leaf samples from healthy and diseased palms through a procedure involving precipitation with polyethylene glycol, phenol and chloroform extractions, fractionation with 2 M LiCl, and further purification with cetyltrimethylammonium bromide. Comparative electrophoretic analysis established that two low-molecular-weight RNAs with the same apparent mobilities in 5% polyacrylamide gels as the diagnostic viroidlike RNAs (ccRNA-1 and ccRNA-2) associated with cadang-cadang disease were correlated uniquely with tinangaja symptoms. A ^{3}H-labeled DNA probe complementary to ccRNA-1 (cDNA) was used to show that the tinangaja-related RNAs have nucleotide sequences equivalent to ccRNA-1. Tinangaja is therefore considered to have the same etiology as cadang-cadang, a disease formerly believed to be restricted to the Philippines. Nucleic acids extracted from coconuts affected with bristle top did not contain such viroidlike RNAs.

Additional key words: molecular hybridization analysis, tatipaka.

Cadang-cadang is perhaps the most serious disease of coconuts (*Cocos nucifera* L.) and has probably killed more palms than all other coconut diseases combined (2). The symptomatology and progress of the disease in affected palms have been studied extensively (2,5,7,18). Two low-molecular-weight ribonucleic acids, ccRNA-1 and ccRNA-2, present only in diseased palms, have properties in common with viroids and are mechanically transmissible to young palm seedlings (8,10,11,13). Avian myeloblastosis virus reverse transcriptase has been used to synthesize ^{3}H-labeled DNA complementary to ccRNA-1 (cDNA), which served as a probe for the detection of ccRNAs in naturally infected African oil palms (*Elaeis guineensis* Jacq.) and buri palms (*Corypha elata* Roxb.) (9).

Tinangaja was reported in coconut as early as 1917 from the island of Guam (17), but similar disorders have not been reported from any other Pacific island. A second report in 1961 named the disease "infectious yellow mottle decline" and described it as closely resembling cadang-cadang (15). This resemblance was later questioned on the grounds that the two diseases were similar only in the induction of sterility and eventual death of the affected palms and that their symptoms appeared similar only when viewed from a distance (2,4,6,7).

Bristle top, undetected in 1917 (17), was reported in the early 1960s from Guam and Anahatan islands of the Marianas (6,15); one author believed it to be the last stage of tinangaja (15). We report investigations comparing these diseases.

MATERIALS AND METHODS

Source of palm material. The coconuts on Guam were surveyed, and samples were collected from the fifth to the seventh youngest fully opened fronds of 14 healthy, eight tinangaja-affected, and two bristle top-affected coconuts. Samples from comparable fronds from palms affected with cadang-cadang were collected in the Philippines (18) and processed in the same way as the samples from Guam.

Nucleic acid extraction. Leaf tissue (50–100 g) was extracted with three volumes of 0.1 M Na_2HPO_4, 10 mM diethyldithiocarbamate, and 0.1% sodium thioglycolate. The slurry was filtered through cheesecloth and mixed with 5% polyethylene glycol 6000 (PEG). The nucleic acids extracted from the PEG-insoluble material were mixed with 90% phenol containing 0.1% 8-hydroxyquinoline (phenol) and concentrated with redistilled ethanol as previously described (8). This material was then dissolved in 1% sodium dodecylsulfate (SDS) and reextracted with one volume of phenol and then with half a volume of chloroform. After ethanol precipitation, the nucleic acids were resuspended in 0.1 M sodium acetate and 1 mM ethylenediaminetetraacetate sodium salt (EDTA), mixed with an equal volume of 4 M LiCl, and left overnight at 0 C. LiCl-insoluble material was recovered by centrifugation and discarded. The soluble fraction was ethanol-precipitated, resuspended in sodium acetate-EDTA, and further purified with cetyltrimethylammonium bromide as previously described (9). Nucleic acids were washed twice in ethanol, dried, and dissolved in appropriate media as specified below.

Polyacrylamide gel electrophoresis. Nucleic acids dissolved in 90 mM tris(hydroxymethyl)aminomethane (TRIS), 90 mM borate, 3 mM EDTA, pH 8.3 (TBE buffer [8]) containing 20% sucrose were fractionated in 1.5-mm 5% polyacrylamide gel slabs buffered in TBE at room temperature for 3–5 hr at a constant current of 10 mA per slab.

Fractionation of nucleic acids on sucrose density gradients. Nucleic acids, suspended in 0.1 × SSC (SSC buffer: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) were fractionated on 10–40% sucrose density gradients (buffered in 0.1 × SSC) by centrifugation at 42,000 rpm for 17 hr in the Spinco SW 50.1 rotor. The central 1–2 ml of each 5-ml gradient, corresponding to the zone containing nucleic acids of approximately the size of the ccRNAs (9), was collected with an ISCO density gradient fractionator. To each fraction, 50 μg of yeast RNA, sodium acetate to 0.1 M, and ethanol to 75% were added. The nucleic acids were collected by

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centrifugation after storage at −20 C for 16 hr, washed in ethanol, dried, and dissolved in 0.12 ml of hybridization buffer (0.18 M NaCl, 0.05% SDS, 0.01 M TRIS-HCl, pH 7.0 [9,12]).

Molecular hybridization analysis (MHA). ^{3}H-labeled DNA complementary to ccRNA-1 (cDNA) was synthesized using S_1-nuclease-cleaved, polyadenylated ccRNA-1 as the template for the avian myeloblastosis virus reverse transcriptase (12).

MHA was carried out essentially as previously described (9,12). Viroidlike enriched fraction aliquots of 0.03 ml of coconut nucleic acids in hybridization buffer were mixed with 0.01 ml (1,000 counts per minute) of cDNA in the same buffer, overlaid with paraffin oil, heated at 100 C for 3 min, then incubated for 25 hr at 65 C.

S_1-nuclease resistance was determined by adding 0.035 ml of the hybridization mixture to 0.4 ml of S_1 assay buffer (0.3 m NaCl, 0.03 M sodium acetate, pH 4.6; and 1 mM $ZnSO_4$, 5% glycerol, pH 4.6) containing 40 μg/ml of denatured calf thymus DNA. Two 0.2-ml aliquots either with or without two S_1-nuclease units (16) were incubated for 40 min at 45 C. Reactions were stopped by adding 1 ml of 10% trichloroacetic acid (TCA) and 75 μg of bovine serum albumin. The ratio of TCA-insoluble radioactivity of aliquots with and without S_1-nuclease was used to determine the percentage hybridization of cDNA to ccRNA-1 in the samples.

RESULTS

Symptomatology. Guam at present has no coconut industry, and palms are almost completely neglected. This and other damage is said to have originated from the indiscriminate use of weed killers (6). Extensive attack and damage by *Brontispa* beetles hamper the study of tinangaja and bristle top, because symptoms can be mimicked, masked, or obscured. Nonetheless, coconut palms with yellowish and reduced crowns, indistinguishable from cadang-cadang-affected trees in the Philippines (2), and others bearing the characteristic tinangaja-mummified nuts (4,6,15,17) were frequently observed throughout the island.

Like cadang-cadang (2,18), tinangaja primarily affects trees 25–30 or more years old. The leaflets of tinangaja-affected coconuts show chlorotic spots very similar to those observed in cadang-cadang-affected trees, although this is not considered a diagnostic symptom.

The similarity of the two diseases, however, has long been questioned on the basis of fruit symptoms, which are, for convenience, used as the principal diagnostic character. In the early stage, cadang-cadang induces the production of smaller-than-normal, rounded nuts scarified around the equator (2,5,7,18), while no such symptom has been reported for tinangaja. Instead, the formation of crinkled, mummified nuts with no kernel inside has been observed (2,4,6,15,17). We have observed two instances of tinangaja-affected palms bearing on the younger inflorescences typically mummified nut clusters but also bearing nuts almost indistinguishable from the typical cadang-cadang rounded fruits on the lowest (oldest) inflorescences (Figs. 1 and 2). The fact that such symptoms previously escaped attention and that they were observed on only two palms across the island may be explained by assuming that they occur only for a short time at that stage of the disease. According to this hypothesis, the mummified nuts occur in the middle stage of the disease, which seems to last the longest. Palms die soon after nut production ceases (4,6,17), whereas in cadang-cadang, sterility is induced much earlier and the late stage (no nuts) can last for years (2,5,7,18,19).

Maramorosch (6) reported that the fronds of coconuts infected with bristle top are much shortened and stiffened and remain erect, thus leaving an upright tuft at the top of the tree. A few palms with these symptoms have been observed (Fig. 3), but the present incidence of this disorder seems to be negligible. The leaflets of the affected trees show juvenile characters—they do not separate normally but remain fused. No mottling appears, and the leaves remain dark green.

Polyacrylamide gel electrophoresis. All but one of the sampled coconut palms with tinangaja symptoms showed two low-molecular-weight RNAs of the same apparent electrophoretic mobilities as the fast-moving variants of the ccRNAs (14), irrespective of the stage of the disease (Fig. 4). Failure to detect cadang-cadang-like RNAs in one tinangaja-affected palm can be explained by a faulty extraction, because no RNAs were detectable in the gel.

Samples were also collected from all available fronds of three palms in the three stages of the disease; they all contained viroidlike RNAs of the same electrophoretic mobilities. The similarity in electrophoretic mobility of the tinangaja-related RNAs contrasts

Fig. 1. A tinangaja-affected palm in the early stage of the disease. Note on the lowest inflorescence a cluster of small, rounded, scarified nuts (black arrows) and on the younger ones clusters of mummified, crinkled nuts (white arrows).

with cadang-cadang, where variants of ccRNAs of different electrophoretic mobilities have been detected in different trees (14) and sometimes even in the same tree (J. S. Imperial, *unpublished*).

No cadang-cadang-like RNAs were detectable in the LiCl-soluble portion of extracts from apparently healthy, insect-damaged, or bristle top-affected coconuts.

Molecular hybridization analysis. The fact that the electrophoretic mobilities of the tinangaja-related viroidlike RNAs appear to be the same as those of the fast-moving ccRNAs variant was not considered in itself unequivocal evidence of the identity of the pathogens involved. We therefore used MHA to determine if specific ccRNA-1 nucleotide sequences could be detected in nucleic acid extracts of tinangaja-affected coconuts.

Table 1 shows the percentage homologies between ccRNA-1 (cDNA) and unfractionated or sucrose density gradient fractionated LiCl-soluble RNA preparations from coconut palms affected by cadang-cadang, tinangaja, or bristle top. RNAs from tinangaja-affected palms showed the maximum percentage hybridization attainable with this system, indicating that they contained nucleotide sequences indistinguishable from ccRNA-1. Furthermore, these common sequences were detected in the low-molecular-weight zone of the sucrose density gradients, where the ccRNAs would be expected to sediment.

TABLE 1. Molecular hybridization of cDNA (ccRNA-1) with nucleic acids extracted from coconut palms infected with cadang-cadang, tinangaja, or bristle top disease

Disease	Experiment	Nucleic acid preparation	Time of hybridization (hr)	DNA hybridized (%)[a]
None	1	No nucleic acid	25	8.6
	2	No nucleic acid	70	2.9
Cadang-cadang	1	Fractionated[b]	25	31.4
	2	Fractionated	70	56.4
Tinangaja	1	Fractionated	25	39.5
	2	Unfractionated[c]	70	76.0
	2	Fractionated	70	67.4
Bristle top	1	Fractionated	25	6.5

[a] Percentage hybridization values have had the "no nucleic acid" value subtracted and show the percentage of input ^{3}H-DNA (ccRNA-1) that forms hybrids.

[b] LiCl-soluble nucleic acids subjected to fractionation on 10–40% sucrose density gradients; fractions containing nucleic acid of approximately the size of the ccRNAs (13) were used.

[c] LiCl-soluble nucleic acids unfractionated on sucrose gradients (see Fig. 4).

DISCUSSION

Since the demonstration of its unique association with diseased palms in the Philippines (8), the viroidlike ccRNA-1 can be used as a diagnostic marker for cadang-cadang disease of coconuts. The discovery that tinangaja-affected palms from Guam contain an

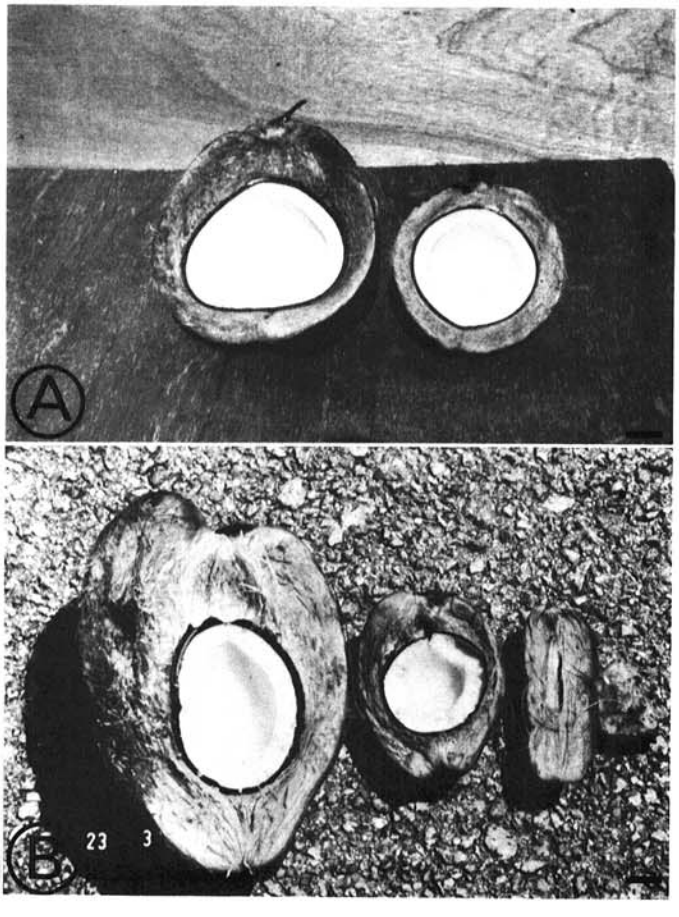

Fig. 2. A, Fruits collected from a palm in the early stage of cadang-cadang disease (right) and from a healthy palm (left) in the Philippines. **B,** Fruits collected from a healthy palm (left), a palm in the early stage of tinangaja disease (center), and a palm in the middle stage of tinangaja disease (right) in Guam. Bars represent 5 cm.

Fig. 3. Coconut affected by bristle top (right), compared with a healthy one (left).

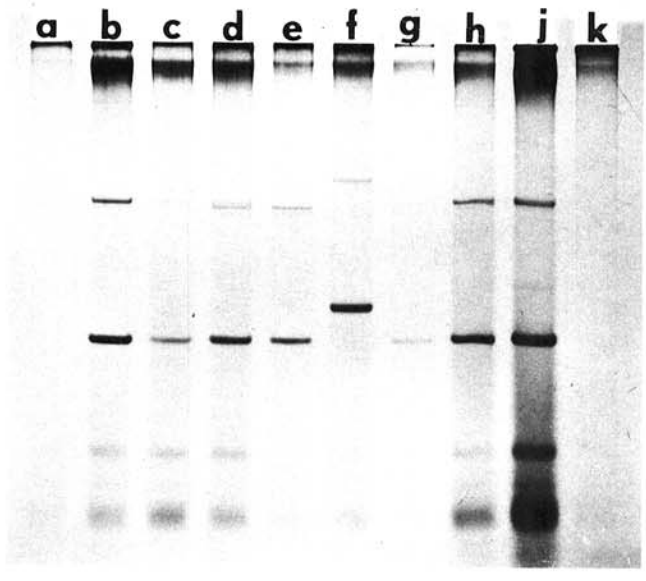

Fig. 4. Polyacrylamide (5% gel slab) coelectrophoresis of nucleic acid extracts of coconuts affected by bristle top (lanes a and k); cadang-cadang (lanes f and g: slow- and fast-moving variants, respectively [10]); and tinangaja in the early (lanes b and c), middle (lanes d, e, and h), and late (lane j) stages of the disease.

essentially identical RNA is convincing evidence that tinangaja and cadang-cadang diseases are caused by the same pathogen. The failure to detect this RNA in normal palms or those affected with bristle top, either by gel electrophoretic analysis or the more sensitive MHA technique (9,12), indicates that tinangaja and bristle top can be considered separate disorders.

Symptomatologies led one author (15) to consider that cadang-cadang and tinangaja were related, but others (2,4,6) have believed them to be different diseases on the basis of nut symptoms. The observation that rounded nuts typical of cadang-cadang can also be found, albeit rarely and possibly briefly, on tinangaja-affected palms suggests that nut symptoms are unreliable for distinguishing these diseases. The different rates of progress of the disease in the Philippines and Guam can possibly be explained by the different cultivars of coconut palms. The cadang-cadang viroid can attack coconut of totally different kinds, such as the "elongated" type on Guam ("wild type" or *niu kafa*) (3) and the more spherically fruited type cultivated in the Philippines ("domesticated type" or *niu vai*) (3).

The disease is seriously threatening a large area in the Philippines (18) and has destroyed the coconut industry on Guam (4,6). Because of the interchanges among the Marianas Islands, the disease may occur in other islands of the South Pacific and thus poses a very serious threat to the area. Symptoms of the disease have in fact been observed on Rota and Tinian, two of the Marianas Islands near Guam (R. G. Beaver, *unpublished*).

Our methods have proven useful in identifying new natural hosts of the cadang-cadang viroid (9); detecting ccRNAs in experimentally inoculated palm seedlings (10; J. S. Imperial, *unpublished*); proving the identity of cadang-cadang and tinangaja, a coconut disease of heretofore obscure etiology; and showing that cadang-cadang is not related to bristle top.

A fourth coconut disease of unknown etiology, known as tatipaka (1), occurs in the East Godavari district of Andhra Pradesh, India. The symptoms of tatipaka are somewhat reminiscent of those of cadang-cadang in that leaflets are deformed and mottled, and of those of tinangaja in that the affected palms produce distorted, narrowed nuts with reduced or no kernel. Tatipaka appears, however, to be unrelated to cadang-cadang, because no low-molecular-weight viroidlike RNAs could be detected by polyacrylamide gel electrophoresis, nor is significant homology with cDNA indicated by MHA (J. W. Randles, *unpublished*).

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