

Transmission of the RNA Species Associated With Cadang-Cadang of Coconut Palm, and the Insensitivity of the Disease to Antibiotics

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ABSTRACT

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Transmission to young coconut seedlings of the RNA species associated with cadang-cadang disease (ccRNA-1 and ccRNA-2) was achieved in two trials using either polyethylene glycol 6000-precipitated material, or nucleic acids extracted from this precipitate, as inoculum. Inoculation was by high pressure injection combined with either rubbing with Carborundum or slashing of petioles with a razor. The appearance of leafspotting symptoms on the young fronds of the RNA-positive inoculated palms between 19 and 22 mo after inoculation suggests that the agent may

have been transmitted as a result of transferring the RNA by the inoculation procedure, and further suggests that the latent period lies between 1.5 and 2.0 yr. It has been confirmed that the cadang-cadang associated RNA species can be detected in the young fronds of palms before symptoms appear, consistent with the view that they are precursors of the disease. The failure of tetracycline and penicillin antibiotics to affect disease progress shows that prokaryotes probably have no primary role in the disease.

Additional key words: yellow mottle decline, growth and productivity of diseased palms, mycoplasma, rickettsia, virus, viroid.

Cadang-cadang or yellow mottle decline is the major disease of coconut (*Cocos nucifera* L.) in The Philippines (2, 13). Although natural spread occurs, the disease agent has not been transmitted experimentally despite numerous attempts (13), and its etiology remains unknown.

The development of the disease follows a predictable sequence, and three main stages are recognized. During the early stage, characteristic non-necrotic yellow leaf spots (9, 17) appear on fronds below the third or fourth frond down from the unopened spear leaf, and nuts temporarily increase in number and become rounded with longitudinal scarifications chiefly around the equator. During the mid-stage, which lasts for several years, spathe, inflorescence, and nut production decline then cease, and leaf spots become more numerous. Palms enter the late stage of disease development when fronds decline in size and number, the leaf spots increase in size and frequency, and pinnae become brittle. The palm eventually dies. Diagnosis of the disease is based largely on its effect on fruit production, and this generally is reliable for field observations because the disease occurs almost exclusively in bearing palms (3). In the rare cases where pre-bearing palms are believed to be diseased, diagnosis is less certain because it is based on the type and distribution of leaf symptoms, general yellow appearance, and stunting; such palms do not bear nuts,

even if they survive to the age of bearing.

Because no nematode, fungal, or bacterial parasite appears to be associated with the disease, a mycoplasma, rickettsia, virus, or viroid is considered to be the most likely cause. Mycoplasma-like organisms, which are associated with the lethal yellowing disease of coconut in the West Indies and Florida (1, 4, 5, 12) have not been detected in the vascular tissue of palms affected by cadang-cadang (Randles, *unpublished*), although the preliminary observation of rickettsialike organisms in the phloem parenchyma of a diseased palm (11) indicates that their possible involvement should be considered. No viruslike particles are associated with the disease (14).

The only clue to the cause of cadang-cadang has been the discovery that two low-molecular-weight species of RNA (ccRNA-1 and ccRNA-2) appear to be associated exclusively with the disease (15). That provided a diagnostic test for cadang-cadang. Furthermore, one of them (ccRNA-1) is structurally similar to the known viroids (15, 16). We now report that the cadang-cadang associated RNA's (ccRNA) have been transmitted experimentally, that prokaryotes appear to have no primary function in the disease, and conclude that a virus or viroid is the most probable cause of cadang-cadang.

MATERIALS AND METHODS

Preparation of inoculum.—The mechanical transmission of virus diseases to woody plants is rarely

successful; but, because in this study the alternative procedure of grafting between palms was not possible, and because no vector of the cadang-cadang agent is yet known, mechanical transmission was the only means available to attempt demonstration of the pathogenicity of a putative virus or viroid agent. Because previous attempts to transmit the pathogenic agent by rubbing sap extracts from leaves of infected palms on the leaves of test palms apparently were unsuccessful (13), we used inocula that had been partially concentrated in their content of the RNA's associated with cadang-cadang. Inocula were taken at different stages in the standard nucleic acid procedure (15), and inoculation was attempted at three different times using different inoculation procedures (see Table 1).

Inoculum A was prepared by blending chopped leaflet tissue in four volumes (w/v) of 0.1 M Na_2HPO_4 containing 0.01 M sodium thioglycollate and 0.1% sodium diethyldithiocarbamate and then clarifying the suspension by low-speed centrifugation. Inoculum B was prepared by mixing inoculum A with nicotine base to 2%. Inoculum C was prepared by blending leaflet tissue with one volume (w/v) of 90% aqueous phenol containing 8-hydroxyquinoline and two volumes (w/v) of 0.05 M Tris-HCl buffer (pH 7.0). After centrifugation, the aqueous phase was extracted three times with ether to remove phenol, and the ether was removed by evaporation at low pressure. Inoculum D comprised polyethylene glycol 6000 (PEG) precipitated material prepared by resuspending the PEG-precipitate (15) from 50 gm of leaflet tissue in 10 ml of 0.05 M phosphate buffer (pH 7.0).

Inoculum E comprised the total nucleic acids in the PEG-insoluble material, prepared by a phenol-SDS extraction, ethanol precipitation, protease digestion, and a further phenol extraction and ethanol precipitation (15). Inoculum was diluted to c. 80 $\mu\text{g}/\text{ml}$ total RNA in Tris (0.089 M) - boric acid (0.089 M) - disodium EDTA (2.5 mM) buffer, pH 8.3 (10). Inoculum F was ccRNA-1, isolated on and recovered from polyacrylamide gels, then precipitated with ethanol, dried (16), and dissolved in the Tris-borate - EDTA buffer to a final concentration of c. 4 $\mu\text{g}/\text{ml}$.

Inoculation.—Coconut palm seedlings were inoculated by high-pressure injection (8, 13) using either a Hypospray® (Scherer Corp., Detroit, MI 48213) injector with an electrically operated air compressor, or a hand-primed Panjet® (Schuco International, London, UK) mechanical hand injector kindly supplied by D. J. Meadows. Deliveries were 0.5 ml and 0.1 ml per injection, respectively. Fibrous sheath material was stripped from the petiole bases to expose the folded white- to pale-green leaflets at the base of the spear leaf. Injections were applied to the petiole or to the outermost of the folded leaflets. Seedlings were 4 or 18 mo old, except in the trial of August 1973 in which inoculum D was injected into the pre-emergent shoot exposed by cutting back the husk of the nut. In preliminary experiments, injection of an aqueous toluidine blue solution gave penetration through at least three leaflets with negligible necrotic damage at the injection site. Two injections were given several centimeters apart, together with either a razor-slashing inoculation (where a stainless safety razor wet with

TABLE 1. Detection of cadang-cadang related RNA species (ccRNA-1) and the development of yellow spots and mosaic symptoms on fronds 1 and 2 of coconut seedlings inoculated with extracts from diseased palms

Inoculation date	Inoculum preparation ^a	Inoculum sources (Number of palms and stages of disease development)	Inoculation ^a procedure	Number of palms		
				Tested	With ccRNA	With ccRNA and symptoms
May 1973	Sap extract - A	Two late-stage palms	"Panjet"	5	0	
	Sap extract - B	Two late-stage palms	"Panjet" or "Hypospray"	10	0	
	Sap extract - C	Two late-stage palms	"Panjet" or "Hypospray"	10	0	
August 1973	PEG precipitate - D	One late-stage palm	"Panjet" and rubbing, repeated at 24 hr on pre-emergent shoots	14	2	2
June 1974	PEG precipitate - D	One mid-stage palm	"Panjet" and razor slashing	20	0	
	Nucleic acid - E	Five mid-stage palms	"Panjet" and razor slashing	20	12	12
	ccRNA-1 - F	One mid-stage palm	"Panjet" and razor slashing	18	0	
	ccRNA-1 - F	One late-stage palm	"Panjet" and razor slashing	20	0	
August 1973 and June 1974	Noninoculated			44	0	

^aInoculum A: leaflet tissue blended in 4 volumes (w/v) of 0.1 M Na_2HPO_4 containing 0.01 M sodium thioglycollate and 0.1% sodium diethyl dithiocarbamate. Inoculum B: inoculum A to which nicotine base was added to 2%. Inoculum C: leaflet tissue blended with an equal volume (w/v) of 90% aqueous phenol and two volumes (w/v) of 0.05 M Tris-HCl buffer (pH 7.0). Inoculum D: PEG 6000-precipitated material (15) resuspended in 0.05 M phosphate buffer (pH 7.0). Inoculum E: total nucleic acids recovered from the PEG-insoluble fraction and suspended in Tris-borate-EDTA buffer (10).

inoculum was used to produce 10 or more parallel cuts across the petiole), or rubbing of the Carborundum-dusted petiole base with a finger wet with inoculum. Inoculated and noninoculated plants were interplanted in the field.

Assessment.—Inoculated seedlings were assayed for ccRNA in February 1976 by harvesting 100 gm of leaflets from fronds 2, 3, or 4, and extracting nucleic acids by the phenol-protease procedure (15). Nucleic acid preparations were analyzed in duplicate by electrophoresis on 3.3% and occasionally 20% polyacrylamide gels (10) with purified ccRNA-1 as a marker in sister gels. Fronds of inoculated and noninoculated seedlings also were examined for leaf spot development.

Chemotherapy with antibiotics.—The effect of antibiotics on the progress of the disease was tested in three trials.

Trial 1.—Tetracycline solutions at 2,000-4,000 $\mu\text{g}/\text{ml}$ were poured directly into three newly bored 2×20 cm holes in the bole of the palm, and these then were stoppered with a cork. This injection technique was shown to be effective in a preliminary experiment by adding aqueous toluidine blue dye to a test hole. The hole was dry within 3 hr, and vascular tissue above and below the hole was stained. Antibiotic solutions likewise were

taken up rapidly, and the observation of phytotoxic desiccation symptoms in the pinnae of some palms injected with heavy doses demonstrated upward systemic movement. Duplicate groups of five palms at early, mid-, and late stages of disease development were used, one set being injected with either tetracycline or tetracycline-HCl. The injection schedule was: 23 May 1973, 1 g injected; at 101 days, 0.25 g; at 166 days, 0.25 g; at 632 days, 0.5 g.

Trial 2.—Thirty-two diseased palms (27 early, four mid-, and one late-stage) were given three injections of tetracycline. The first injection was applied under gravity through a polyethylene tube inserted tightly into a hole in the petiole of fronds 3, 5, and 7. One liter, at a concentration of either 625, 1,250, or 2,500 $\mu\text{g}/\text{ml}$, was injected into each petiole. Each concentration was applied to each of the three fronds on each palm, with treatment positions being varied from palm to palm to allow all combinations of concentration with frond age. A total of 4.375 g was injected into each palm. Injections at all concentrations induced toxic symptoms in the distal pinnae within 3 mo. The second and third injections then were made as in Trial 1, 3 mo apart, injecting 1.75 g per palm as a 7,000 $\mu\text{g}/\text{ml}$ solution. Fourteen diseased palms were left untreated.

TABLE 2. Detection of cadang-cadang related RNA ccRNA-1 in fronds 1 to 12 of twelve diseased coconut palms

	Frond position below spear leaf											
	1	2	3	4	5	6	7	8	9	10	11	12
Number of palms with ccRNA-1	7 ^a	9	9	10	9	11	11	12	12	11	12	12

^aNumber of palms from the 12 assayed containing detectable ccRNA-1 in the frond position indicated.

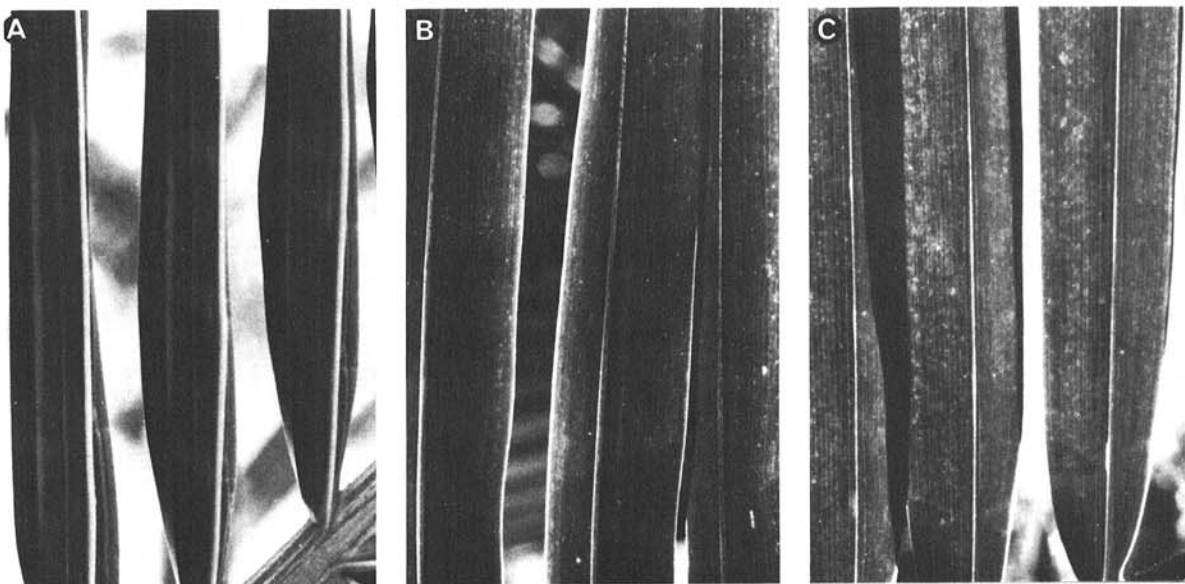


Fig. 1—(A to C). Non-necrotic yellow leaf-spotting on leaflets on the first frond below the spear leaf (B), and yellow spotting with a mild mosaic on the second frond (C) of an inoculated coconut seedling containing ccRNA, compared with the second frond of a noninoculated seedling (A). Photographs taken 19 mo after time of inoculation.

Trial 3.—Ten palms (six early, two mid-, two late-stage) were given two injections each of 1.75 g of penicillin (as a 7,000 $\mu\text{g}/\text{ml}$ solution 4 mo apart, using the technique of Trial 1.

The effect of antibiotics on disease progress was assessed by describing the stage of disease development, and measuring the rate of frond production, and the numbers of spathes and nuts produced during and after treatment. Rate of frond production was determined by marking the spear leaf at zero time and measuring the rate at which it changed position in the crown of the palm with time.

RESULTS

Detection of ccRNA in inoculated coconut palms.—Ribonucleic acid ccRNA-1 was extracted from inoculated seedlings in two of the transmission trials (Table 1); in one trial PEG-insoluble material was used as the inoculum, and in the other the inoculum was the combined nonfractionated total nucleic acids. Ribonucleic acid ccRNA-2 (15, 16) also was detected with ccRNA-1, but it was not always easily distinguishable from normal palm RNA on the basis of its electrophoretic mobility. None of the noninoculated palms tested contained ccRNA-1, indicating that the ccRNA was detected as a result of inoculation.

At the time of the assays, 19 mo after inoculation, 10 of the 14 palms with ccRNA-1 were showing bright yellow spots on the first frond down from the spear leaf, and spots usually together with a yellow green mosaic on the

second frond down (Fig. 1). At 22 mo, all 14 were showing spots and a mild mosaic. Although noninoculated palms occasionally showed spots, and sometimes a mottle, the characteristic feature of spots on the inoculated palms was that they were on younger fronds than was observed with the noninoculated palms. The detection of ccRNA-1 therefore appears to be associated with the appearance of a mild symptom on the youngest opened frond.

Detection of cadang-cadang related RNA species (ccRNA) before symptoms appear.—The results above with young inoculated palms suggest that ccRNA can be detected before leaf symptoms appear. This phenomenon also was demonstrated in a trial in which 12 mature palms representing all stages of disease were assayed for the presence of ccRNA-1 in fronds 1 to 12. Ribonucleic acid ccRNA-1 frequently was detected in the symptomless fronds 1, 2, and 3 (Table 2), that is, up to 2 months before initial symptoms appear. The frequency of detection increased after the appearance of symptoms on fronds 4 and older, with maximum reliability of detection in fronds older than frond 5.

Failure of antibiotics to alter progress of disease.—The effect of cadang-cadang disease on the growth and productivity of coconut palms is illustrated in Tables 3 and 4. Table 3 shows the results of Trial 1, which show that the mean number of spathes, inflorescences, and bunches of nuts, and the mean rate of frond production is progressively lower in palms at increasingly advanced stages of the disease. Three tetracycline injections had no significant effect on any of these parameters when

TABLE 3. Effect of treatment with tetracycline on the progress of cadang-cadang disease in palms at the early, mid-, and late stages of disease at the commencement of treatment (Trial 1)^a

Tetracycline	Stage of disease at commencement of treatment							
	Healthy		Early		Mid-		Late	
	-	+	- ^c	+	-	+	-	+
Mean number of spathes ^b	2.8 $\pm 2^c$	3.0 ± 0	... ^f	2.0 ± 6	1.2 ± 5	1.4 ± 4	0	0.2 ± 2
Mean number of inflorescences ^b	1.4 ± 5	0.6 ± 6	...	2.6 ± 8	1.2 ± 7	0.8 ± 2	0	1.0 ± 1
Mean number of bunches of nuts ^b	10.6 ± 9	10.6 ± 5	...	4.8 ± 2.0	0.6 ± 5	1.4 ± 7	0	0
Mean rate of frond production ^c	15.4 ± 5	13.6 ± 4	...	11.9 ± 2	11.1 ± 5	11.9 ± 4	8.2 ± 6	10.4 ± 4
Stage of disease development ^d								
Palm 1	H ^g	H	...	M	D	D	L	D
2	E	H	...	M	D	L	L	L
3	H	H	...	E	L	L	L	L
4	H	H	...	M	D	M	L	L
5	H	H	...	M	L	M	D	L

^aFive palms were included in each treatment group, and disease progress was assessed by measuring the mean number of spathes, inflorescences, and bunches of nuts; the mean rate of frond production; and the progression of disease from one stage to another.

^bParameters measured 9 mo after treatment commenced.

^cNumber of fronds produced per year.

^dDetermined 36 mo after treatment commenced.

^eStandard error.

^fThe symbol ... indicates not tested.

^gAbbreviations: H = healthy, E = early stage, M = mid stage, L = late stage, and D = dead.

measured 9 mo after commencement of the treatment, and approximately 3.5 mo after the last injection. Palms treated in the late stage showed a slightly higher rate of frond production than the untreated group, but this was still less than in all other stages. After 36 mo, and following another injection, no treated palms showed regression of disease.

When the treated palms were mainly at the early stage at the commencement of the trial (Trial 2), tetracycline injections failed to prevent the progression of a proportion of the palms to more advanced stages of disease, nor did they affect significantly the mean number of fronds, spathes, or nuts (Table 4).

The effect of penicillin treatments was assessed for a shorter period on a smaller number of palms (Table 4). No apparent improvement in injected palms was observed.

DISCUSSION

Our results indicate that ccRNA-1 has been transmitted to coconut seedlings in two of the trials described. The appearance of yellow leafspots on the youngest opened fronds of the palms containing ccRNA-1, leads us to suggest that the transfer of ccRNA-1 coincides with the transfer of the causal agent of cadang-cadang. This conclusion must remain tentative pending

the observation of the inoculated palms over a number of years for the development of the characteristic field symptoms, but it seems probable that if ccRNA-1 is a diagnostic marker for cadang-cadang (15), its isolation from inoculated palms indicates that the disease agent has been transmitted. The minimum period from inoculation to the appearance of the first suspected leaf symptoms is estimated to be between 19 and 22 mo. It is noteworthy that the youngest opened frond of the inoculated seedlings developed leafspots, whereas in older palms, the spots appear on fronds older than frond three or four.

The failure to detect transmission in the other trials, including that in which ccRNA-1 was used as inoculum, may be due to any of a number of factors, such as source, stability, and method of preparation of inoculum; time, site, and mode of inoculation; and susceptibility of test seedlings. The optimum conditions for transmission have yet to be ascertained, but the important features emerging from the results described include: the partial concentration of the inoculum, the combination of nucleic acids from several sources as inoculum, and the use of high pressure injection (8) and razor-slashing (2) inoculation techniques.

We consider it most improbable that ccRNA-1 was detected in inoculated palms as a result of natural transmission. None of the extracts from the noninoculated controls tested contained ccRNA-1,

TABLE 4. Change in the stage of disease development and the mean number of fronds, spathes, and nuts on cadang-cadang diseased palms with time, and the effect of tetracycline (Trial 2) and penicillin (Trial 3) injections

Disease stage and other disease-rating parameters	Treatment	Palms (no.) and disease-indicator parameters (mean no.) observed:		
		June 1975	December 1975	April 1976
Number of palms at early stage	U ^a	13	8	8
	T	27	20	12
	P	...	6	6
Number of palms at mid-stage	U	0	6	6
	T	4	11	17
	P	...	2	2
Number of palms at late stage	U	1	0	0
	T	1	1	3
	P	...	2	2
Mean number of fronds	U	31.6±1.6 ^b	31.5±1.5	29.6±1.8
	T	32.4±0.7	32.1±0.7	28.7±0.8
	P	...	28.9±2.2	29.1±2.0
Mean number of spathes	U	2.4±0.1	1.9±0.2	2.1±0.2
	T	2.7±0.1	2.1±0.1	2.1±0.1
	P	...	1.6±0.3	2.3±0.3
Mean number of nuts:				
	Total			
	U	64±16	24±7	14±5
T	72±7	40±5	20±4	
P	...	32±10	32±11	
Harvestable	U	11±2	7±1	4±1
	T	14±2	10±2	4±0.5
	P	...	3±2	3±1

^aAbbreviations: U = untreated, T = tetracycline treatment, and P = penicillin treatment. Tetracycline was injected in June, August, and December, 1975. Penicillin was injected December 1975 and April 1976. (Refer to Trials 2 and 3, Materials and Methods.)

^bStandard error.

indicating a rate of natural infection no higher than 2.3%, and if data from trials in which no ccRNA-1 was detected also are taken into account, natural infection is calculated as no higher than 0.8%. Furthermore, the incidence of the disease in palms between 1 and 5 yr old in the field is estimated at 0.025% (19). The rates obtained in the two successful trials, in which the seedlings were assessed at the age of approximately 3 yr, are many times that expected through natural infection.

The "viroid hypothesis" for cadang-cadang is based on the structural similarity of ccRNA-1 to the known viroids (16, 18), although it differs from normal plant RNA of the same approximate size (16). Proof of a viroid or virus hypothesis depends upon demonstrating the pathogenicity of the presumed agent. The discovery of a means of transmitting the disease marker, and possibly the pathogen, now provides a means of determining the nature of the causal agent of cadang-cadang.

Two lines of evidence suggest that mycoplasma-like or rickettsialike organisms do not induce cadang-cadang. The ability to transmit the disease agent mechanically with a nucleic acid preparation is a feature of virus or viroid diseases and is unknown for pathogens of the prokaryote group; and the insensitivity of the disease progress, and absence of any regression following tetracycline (7) or penicillin treatment (20) leads us to discount these organisms as possible causes of cadang-cadang. It is noteworthy that the tetracycline dosage and duration of treatment exceeded that given to produce recovery in lethal yellowing diseased palms in Florida (4, 5). The method of injection used here was similar to that of McCoy (4) and was demonstrably effective here in obtaining rapid uptake. Nevertheless, the possible involvement of prokaryotes in the late stages of the disease as secondary pathogens cannot be excluded, and the slightly greater rate of frond production in the tetracycline-injected late stage palms compared with the noninjected, could suggest that a mycoplasma-like organism affects palms at this stage of the disease. Conversely, this result also could be explained by the much poorer weed control around the noninjected palms, leading to a depressing effect on growth rate additional to that due to the disease. The lack of a marked response to tetracycline like that obtained with lethal yellowing disease at least supports the view that cadang-cadang has a different etiology from lethal yellowing (6), a disease of suspected mycoplasma etiology.

Observations on the growth of diseased palms (Table 3) give a quantitative comparison of the previously reported effects of cadang-cadang on spathe, inflorescence, and nut production, and show the dramatic losses resulting from the disease. It is apparent that the progressive reduction in the number of fronds in the crown of diseased palms can be explained by a reduced rate of frond production, rather than an increased rate of frond senescence.

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