Detection and Concentration of Coconut Cadang-Cadang Viroid in Coconut Leaf Extracts

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ABSTRACT

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Procedures for the detection of coconut cadang-cadang viroid (CCCV) by polyacrylamide gel electrophoresis (PAGE) and molecular hybridization with ^{3}H- or ^{32}P-labelled complementary DNA (cDNA) are compared. The lower limits of detection per assay for the three techniques were: PAGE, 50 ng; ^{3}H-cDNA hybridization in solution, 0.5 ng; ^{32}P-cDNA spot hybridization on nitrocellulose membranes, 0.25 ng. Spot hybridization was considered the best for routine diagnosis of CCCV because of its sensitivity, ease of operation, and reliability. Infection in inoculated seedlings could be detected by this method at least 4 mo before the viroid band was detectable by PAGE. Concentrations of CCCV in partially purified nucleic acid extracts were estimated by molecular hybridization and measurements of yields of purified viroid. In the same palm, young fronds contained lower amounts of CCCV than mature fronds. Palms in the early and medium stages of disease contained similar amounts of CCCV while late stage palms contained lower amounts. Palms at the same stage of disease from varied geographical locations with different levels of disease incidence contained comparable levels of CCCV.

Cadang-cadang, a serious disease of coconuts in the Philippines, has caused widespread damage in the past 60 years (2) and although its economic importance has declined in recent years, the disease still kills over 500,000 trees a year (17). A low-molecular-weight viroidlike RNA (ccRNA) is associated with cadang-cadang diseased palms (13). These RNAs have been characterized and shown to differ in size, but not in sequence complexity (7). The smallest of the ccRNAs, ccRNA-1 with a molecular size of 246 residues, has been shown to be the basic infectious unit (N. A. Mohamed, *unpublished*) and can therefore be regarded as the coconut cadang-cadang viroid (CCCV). The disease has been routinely diagnosed by the detection of the characteristic ccRNAs by polyacrylamide gel electrophoresis (PAGE) (15). This method is reliable for the detection of ccRNAs in field samples where these RNAs occur in high concentration. A more sensitive method was needed, however, for the rapid detection of ccRNA in inoculated seedlings in resistance screening and infectivity tests.

A complementary DNA (cDNA) probe can be used as a sensitive diagnostic tool for detecting ccRNAs in infected palms (14). A

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cDNA probe has been used for the routine detection of avocado sunblotch viroid, and the technique is more sensitive than PAGE for detecting the viroid (12). The usefulness of this technique was extended by hybridizing the cDNA probe with viroid RNA that had previously been attached to nitrocellulose filters (10). This allowed rapid processing of large numbers of samples.

The first part of this study was therefore undertaken to compare three methods for detection of CCCV (PAGE, ^{3}H-cDNA probe in solution, and spot hybridization with a ^{32}P-cDNA probe) and to determine their potential as diagnostic tools in inoculation trials.

In the second part of this study, concentrations of CCCV in coconut leaf extracts were estimated by hybridization analysis and yield measurements of purified viroid. Cadang-cadang disease offers a unique system for studying variations in amounts of a viroid with respect to age of leaves, geographical location, and disease incidence. The position of leaves on a coconut palm is directly related to their age (9) while the history and epidemiology of the disease in the Philippines has been studied in detail (2,17). Therefore, it was possible to compare viroid levels in fronds of different ages on the same palm, in palms at different stages of disease, and in palms from different areas. These factors were considered important not only in designing a diagnostic screen procedure, but also in epidemiological studies.

MATERIALS AND METHODS

Source of infected material. Leaf samples taken from naturally infected coconut palms were used as a source of ccRNA for purification; samples from both naturally infected and mechanically inoculated palms were used in routine hybridization assays.

Purification of ccRNAs. For routine assays by PAGE or molecular hybridization, partially purified nucleic acid extracts were prepared as described earlier (8); 10-g samples were extracted and the final nucleic acid pellet was resuspended in 50 μl of sterile distilled water.

For cDNA synthesis and yield measurements, nucleic acids were extracted from infected palms and circular ccRNA-1 was purified from these extracts by three cycles of preparative PAGE as described by Mohamed and Imperial (*unpublished*). Yields of purified CCCV were estimated by measuring the $A_{260\ nm}$ on a spectrophotometer and calculating the RNA concentration by using an extinction coefficient of $E^{0.1\%}_{260\ nm} = 25$.

Preparation of complementary DNA (cDNA) to CCCV. Complementary DNA to CCCV, labelled either with ^{3}H-dCTP, specific activity 61 Ci/mmol, (for hybridizations in solution) or α-^{32}P-dGTP, specific activity 200 Ci/mmol (for spot hybridization), was prepared essentially as previously described (14). Purified circular ccRNA-1 was treated with S_1 nuclease (two units of enzyme per μg of RNA for 30 min at 45 C) to cleave the molecule at its single-stranded regions. Polyadenylation of the exposed 3'-termini was carried out using ^{3}H-ATP (5) and cDNA was prepared by using the polyadenylated S_1-nuclease-treated ccRNA-1 as a template (14). Incorporation of label into cDNA was 1.24×10^6 cpm per 2 μg of ccRNA for ^{3}H-cDNA and 10^7 cpm per 2 μg of ccRNA for ^{32}P-cDNA.

Hybridization studies. Hybridizations in solutions using ^{3}H-cDNA were carried out as previously described (14). In each assay, 1,200 cpm of probe were used. Samples were incubated at 65 C for 72 hr and the extent of hybrid formation was estimated by measuring the resistance to digestion by S_1 nuclease (6). Values were corrected for self-annealing (4) and expressed as percent hybrid formation. Zero time hybridizations were included in each assay to check for the presence of inhibitors in the nucleic acid extracts that may interfere with the assay with S_1 nuclease (12).

Spot hybridizations using ^{32}P-cDNA probes were carried out on nitrocellulose membranes (3,10) cut into 3 × 2-cm pieces and marked with a pattern of 6-mm-wide squares (15 per piece). Membranes were pretreated as described by Thomas (16) and 2-μl aliquots of the test samples were spotted on each square. Twelve test samples, one control positive (nucleic acid extracts from a diseased tree) and one control negative (nucleic acid extracts from a disease-free tree), were spotted on each piece of membrane. The membranes were then dried in vacuum at 80 C for 2 hr and incubated in 100–200 μl/cm^2 of prehybridization buffer (16) for 20 hr at 50 C in a sealed plastic bag. This buffer was then removed and replaced with a similar volume of hybridization buffer (16) containing about 2×10^4 cpm of ^{32}P-cDNA; the membranes were incubated for a further 24 hr at 50 C. At the end of this period, the membranes were washed (16), blotted dry, placed between two layers of Saran wrap, and autoradiographed at 22 C for 18–36 hr on Kodak X-omat film.

RESULTS

Comparison of three methods for detection of CCCV. Sensitivities of the three methods used to detect CCCV - PAGE, ^{3}H-cDNA hybridization in solution, and ^{32}P-cDNA spot hybridization on nitrocellulose membranes were compared by serial dilution of a purified preparation of ccRNA-1 and estimating the end point of detection by the three methods.

For PAGE, 25-μl aliquots of nucleic acid extracts, containing CCCV ranging in concentration from 64 μg/ml to 250 ng/ml, were loaded on to 0.75-mm-thick 5% gels and electrophoresed for 2.5 hr at 25 mA before staining with 0.05% toluidine blue. The end point of detection was an RNA concentration of 2 μg/ml, corresponding to a loading of 50 ng of CCCV per gel in a 25-μl sample volume. Therefore, the minimum amount of CCCV detectable by this method was 50 ng per assay.

For hybridizations in liquid with a ^{3}H-cDNA probe, 10-fold serial dilutions of purified CCCV were prepared with concentrations ranging from 10 μg/ml to 0.1 ng/ml. Five microliter aliquots of the diluted solutions were hybridized with the probe in an assay volume of 50 μl. The results showed that hybridization to a value half that of the plateau level of 70% was obtained at a nucleic acid concentration of 100 ng/ml in the original sample; a further 10-fold dilution gave a hybridization level of only 15%. Therefore, the minimum amount of CCCV detected by this method was 0.5 ng per assay.

Spot hybridizations with ^{32}P-cDNA were carried out by dilution of the purified CCCV in a fourfold series from 32 μg/ml to 125 pg/ml. Two-microliter aliquots of each dilution were spotted on nitrocellulose membranes and hybridized for 24 hr against the ^{32}P-cDNA probe before autoradiography. The end point of detection was 125 ng/ml (Fig. 1). Therefore, the minimum amount of CCCV detectable by this method was 0.25 ng per assay.

In routine assays, nucleic acids from 10 g of leaf tissue were resuspended in 50 μl of solution. This was the minimum volume that would fully resuspend the extract and avoid pipetting problems because of viscosity. Aliquots of the nucleic acid solution were then used for detection of CCCV: 25 μl (from 5 g of leaf) for PAGE, 25 μl (from 5 g of leaf) for liquid hybridization, and 2 μl (from 0.4 g of leaf) for spot hybridization. These sample sizes were standardized as being the most practical for our laboratory. Under these conditions, the minimum concentrations of CCCV detectable in coconut leaf extracts by the three methods were (expressed as ng of CCCV per gram of fresh leaf): PAGE, 10 ng/g; liquid hybridization, 0.1 ng/g; and spot hybridization, 0.5 ng/g. These values are dependent on the sample size and can be altered by changing the volume of nucleic acid extract used for each assay. However, the sensitivities of the three methods, when expressed as nanograms of CCCV detected per assay, are independent of sample size and can therefore form the basis for a comparison of the three techniques.

Detection of CCCV in inoculated seedlings. The relative sensitivities of PAGE and spot hybridization assays were compared by using these methods to detect CCCV in inoculated seedlings. In a series of inoculation trials, 165 coconut seedlings were inoculated with partially purified ccRNA between September and December 1980. The seedlings were analyzed for presence of CCCV by spot hybridization in November 1981 and by PAGE in November 1981 and March 1982. All the seedlings that showed a viroid band on gels in November 1982 were found to be positive by spot hybridization

(Table 1). Seven seedlings found to be positive by hybridization but negative by PAGE in November 1981 were found to be positive by PAGE 5 mo later. A third of the seedlings were positive by hybridization, but were negative by PAGE at the last sampling available (March 1982). The viroid may be detected by PAGE in these seedlings at a later date. Some seedlings that were negative by hybridization and PAGE at the first sampling were found to be positive by PAGE at the second sampling.

Concentration of CCCV in leaf extracts. The concentrations of CCCV in partially purified nucleic acid extracts from coconut leaves were estimated by hybridization analysis with ^{3}H- or ^{32}P-labelled cDNA probes and compared with yields of CCCV purified from coconut leaves. Samples were collected from different fronds of the same palm, from palms at different stages of disease from the same area, and from palms at the same stage of disease from different areas of the Philippines.

The variation in concentration of CCCV in different fronds of the same palm was determined by sampling selected fronds starting with the youngest open frond (#1) and ending with a 2-yr-old frond (#27). The results from a palm in the early stage of the disease containing the fast form of CCCV (9) are shown in Table 2. The youngest fronds contained the lowest amount of CCCV and the mature fronds the highest. These results were confirmed by purifying the circular form of ccRNA-1 from young (1- to 4-mo-old) and mature (15- to 18-mo-old) fronds from two palms in the early stage of disease caused by the fast form of CCCV. The yields from the young fronds were 72 and 60 ng of CCCV per gram of fresh leaf and from the mature fronds, 153 and 106 ng/g, respectively.

The concentrations of CCCV in palms at different stages of disease were compared by sampling frond 12 from 30-yr-old palms in the same plantation. Nucleic acids were extracted from palms in the early, medium, and late stages of disease development and the CCCV concentrations were estimated by serial dilutions of the extracts and spot hybridization to determine the end point of detection (Fig. 1B). Palms in the early (fast form of ccRNA) and medium stage (slow form of ccRNA) contained comparable amounts of ccRNA (128 ng/g), while those in the late stage contained lower amounts (32 ng/g).

These results were compared with yields of CCCV purified from 12-mo-old fronds of palms in the early, medium, and late stages of disease. The yields of CCCV from palms in the early and medium stages of disease were comparable (Table 3). There was a decline in yield from late-stage palms, but this was not significant ($P = 10\%$).

The concentration of CCCV in nucleic acid extracts from palms in different areas was compared by sampling frond 12 of one palm in the medium stage of disease from each of three areas on Luzon island: Sorsogon, at the southern end of the cadang-cadang infected area; Camarines Sur, at the center of this area; and Infanta, Quezon, from the northern limit of the diseased area. The

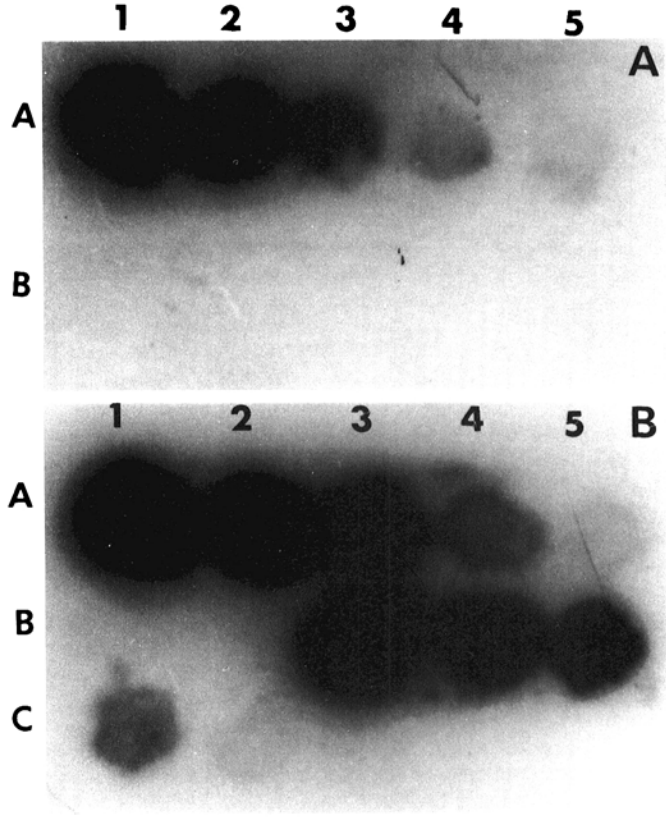

Fig. 1. Spot hybridization of CCCV-^{32}P-cDNA to CCCV. **A,** Fourfold dilutions of purified ccRNA were hybridized with ^{32}P-cDNA after binding to nitrocellulose membranes and autoradiographed for 16 hr. The concentrations of ccRNA were as follows: Row A 1–5, 32 μg/ml, 8 μg/ml, 2 μg/ml, 0.5 μg/ml, and 125 ng/ml, respectively; Row B 1–5, 32 ng/ml, 8 ng/ml, 2 ng/ml, 0.5 ng/ml, and 125 pg/ml. **B,** Fourfold dilutions of nucleic acid extracts from an early stage (Row A 1–5 and Row B 1–2) and a medium stage (Row B 3–5 and Row C 1–5) diseased palm. After binding to the membranes, the nucleic acids were hybridized with ^{32}P-cDNA and autoradiographed. The end points of detection were 1:256 for both palms. The dilutions were as follows: Row A 1–5, undiluted sap, 1:4, 1:16, 1:64, and 1:256; Row B 1–2, 1:1,024 and 1:4,096; Row B 3–5, undiluted sap, 1:4, and 1:16; Row C 1–5, 1:64, 1:256, 1:1,024, and 1:4,096.

TABLE 1. Detection of coconut cadang-cadang viroid (CCCV) in inoculated seedlings by PAGE and spot hybridization with a ^{32}P-cDNA probe

Category	Assay method: PAGE Nov 1981	PAGE Mar 1982	cDNA probe Nov 1981	Seedlings tested[a] (no.)
1	+[b]	+	+	26
2	−[b]	+	+	7
3	−	−	+	55
4	−	+	−	4
5	−	−	−	73

[a] Total number of seedlings inoculated = 165.
[b] Indicates that CCCV was detected (+) or not detected (−) by the method specified at a particular time.

TABLE 2. Estimation of the concentration of coconut cadang-cadang viroid (CCCV) in nucleic acid extracts of different fronds from an early stage diseased palm by spot hybridization with a ^{32}P-cDNA probe

Frond no.	Age of frond (mo)	End point of detection[a]	Concentration of CCCV in extracts[b] (ng/g fresh leaf)
1	1	1:16	8
6	6	1:64	32
12	11	1:256	128
20	18	1:256	128
27	24	1:64	32

[a] Extracts were diluted in a twofold series and the end point of detection was determined by spot hybridization.
[b] Determined from the end point of detection by comparison with the minimum amount of CCCV (250 pg) detected in the same experiment using purified viroid.

TABLE 3. Estimation of yields of coconut cadang-cadang viroid (CCCV) purified from 12-mo-old fronds of 30-yr-old palms at different stages of disease

Stage of disease	Trees sampled (no.)	Form of ccRNA	Yields of CCCV (ng/g)
Early	8	Fast	97 ± 31
Medium	8	Slow	91 ± 31
Late	3	Slow	50 ± 24

[a] CCCV was purified from 1.0 to 2.0 kg of leaf material from frond 12 and final yields of the purified viroid were estimated by measurement of the concentration of circular ccRNA-1.

concentration of CCCV in each sample was estimated by hybridization analysis with a ^{3}H-cDNA probe in solution and determining the $Rot_{½}$ values; a $Rot_{½}$ value of 10^{-2} mol·sec·L^{-1} for purified CCCV obtained in the same experiment was used to determine the percentage by weight of ccRNA in the nucleic acid extracts (11). Although there was some variation between different isolates in the proportion of ccRNA in the nucleic acid extracts, the total amounts of ccRNA extracted from leaves were comparable for the three isolates and there was no correlation with disease incidence (Table 4).

These results were augmented by an extensive analysis of yields of CCCV purified from palms in the early and medium stage of disease from different parts of the cadang-cadang infected area. Three palms were sampled in each area by collecting frond 12 from each palm and extracting nucleic acids from 1.5 to 2.0 kg of leaf material. CCCV was purified by three cycles of PAGE and yields of the purified circular form were estimated. The results show that although there was variation in the amounts of CCCV extracted from different palms, there was no apparent correlation with geographical distribution or disease incidence (Table 5). The only area that showed a major difference was Infanta where yields were significantly lower. However, subsequently it was found that this was a result of storage conditions in transit. Due to the isolation of the area, samples were kept at ambient temperatures (25–30 C) for 2 days and this may have resulted in degradation of the viroid. The concentration of ccRNA in the Infanta sample, estimated by hybridization analysis, which measures all ccRNA-related sequences including fragments of nucleic acids, was similar to that in other samples (Table 4). However, the yield of purified CCCV, intact circular ccRNA molecules, from the Infanta samples was lower than yields from other areas (Table 5). These observations would support the suggestion that degradation of the intact viroid RNA occurred in transit.

TABLE 4. Estimation of the concentrations of coconut cadang-cadang viroid (CCCV) in nucleic acid extracts from coconut palms from three areas of the Philippines by hybridization analysis with a ^{3}H-cDNA probe

Area	Incidence of cadang-cadang[a] (%)	$Rot_{½}$ value (moles·sec·liter^{-1})	CCCV concentration[b] as percent of nucleic acid	CCCV concentration[b] ng/g[c]
Bacon, Sorsogon	7	0.603	1.65	89
Tinambac, Camarines Sur	25	1.175	0.85	82
Infanta, Quezon	20	0.933	1.07	111

[a] Expressed as percentage of palms showing cadang-cadang symptoms in the area.
[b] Calculated using a $Rot_{½}$ value of 10^{-2} mol·sec·liter^{-1} for purified CCCV.
[c] Expressed as nanograms of ccRNA per gram fresh leaf.

TABLE 5. Yields of coconut cadang-cadang viroid (CCCV) from three cadang-cadang infected palms from each of six different regions of the Philippines

Sample	Incidence of cadang-cadang (%)	Yields of ccRNA-1 in μg/kg[a] for tree number: 1	2	3
1. Albay	1	54 (s)[b]	67 (s)	134 (f)
2. Sorsogon	1	68 (s)	128 (s)	60 (f + s)
3. Camarines Sur	20	59 (s)	59 (f)	33 (s)
4. Atimonan	Few isolated trees	43 (s)	70 (s)	38 (s)
5. Bondoc	Few isolated trees	132 (s)	44 (s)	94 (s)
6. Infanta	10% in localized area	17 (s)	9 (f)	20 (s)

[a] CCCV was purified from 1.5–2.0 kg of leaf tissue from frond 12 of each tree and the yield of purified circular ccRNA-1 was estimated with a spectrophotometer.
[b] CCCV forms: (s) indicates the slow form and (f) the fast form.

This suggestion was further tested by comparing yields of CCCV from palms from isolated areas with yields from palms from readily accessible areas. Samples from the former areas were in transit for about 2 days while those from neighboring areas were placed under refrigeration within 6 hr of collection. The mean yield of CCCV from the isolated areas (eight samples) was 18 ± 7 ng/g fresh leaf and for the accessible areas (eight samples) was 81 ± 35 ng/g fresh leaf; these were significantly different ($P = 0.1\%$).

DISCUSSION

Our results show that detection of CCCV in nucleic acid extracts from coconut leaves by molecular hybridization in solution with a ^{3}H-cDNA probe or spot hybridization with a ^{32}P-cDNA probe is at least 100× more sensitive than by PAGE. These results are in agreement with those obtained for avocado sunblotch viroid (ASBV) (1,11) and potato spindle tuber viroid (PSTV) (10). However, PAGE is suitable for detection of CCCV in naturally infected field samples where the concentrations of ccRNAs are usually well above the minimum levels detectable by this method and where it is necessary to determine which electrophoretic form (9) of ccRNA is present. Molecular hybridization with a cDNA probe is more useful as a diagnostic tool in assaying infectivity and resistance trials where sensitivity and time are more critical. Our results show that the use of spot hybridization can shorten assay times by at least 4 mo. One factor to be borne in mind when comparing PAGE and hybridization as detection methods is that while the former only detects intact CCCV, the latter detects all CCCV-related sequences. This would increase the sensitivity of the hybridization method. However, in our experiments, purified preparations of CCCV were used to compare sensitivities of the three methods and therefore comparison of these results is valid.

Although molecular hybridization in solution is as sensitive a detection method as spot hybridization, under our conditions the latter method has a number of advantages. Hybridizations in solution require the use of a scintillation counter, which is difficult to maintain in the isolated conditions in which the laboratory operates, while spot hybridizations do not require elaborate equipment. This technique allows large numbers of samples to be processed rapidly and with ease (10); this is important for routine indexing of inoculation trials where several hundred samples may be processed every week. The sensitivity of the spot hybridization method can be further improved by using a ^{32}P-cDNA probe with a high specific activity obtained by nick translation of cloned CCCV-cDNA (10). Experiments to do this are under way and a cloned probe should be available soon for routine diagnostic work. The sensitivity of the spot hybridization method can also be improved by carrying out the autoradiography step with the aid of intensifying screens at −70 C or by prolonging the exposure time.

In the second part of this study, concentrations of CCCV in coconut palms were determined by molecular hybridization and by measurement of yields of purified viroid. Younger fronds contained lower amounts of ccRNA than mature fronds on the same palm, indicating that mature fronds would be the most suitable source of material both for diagnosis and extraction of viroid RNAs. The lowest level of ccRNA was found in the very young fronds which were still developing and in the old senescing fronds. Replication of ccRNA occurs in the young fronds as the concentration of ccRNA in these fronds increases as they mature. The lower level of ccRNA in the old leaves may be due either to in vivo degradation because of senescence or to in vitro degradation during extraction as a result of higher levels of phenolic compounds in senescing leaves. The variation in concentration between the youngest frond and mature fronds is about 15-fold and is age-related as concentration of CCCV increases with age and reaches a peak in mature fronds. This is in marked contrast to ASBV-infected trees where leaves from different branches of the same tree can show a 1,000-fold variation in concentration of ASBV (1).

There was no significant variation in concentration of CCCV between different trees at the same stage of disease or even between palms at different stages of disease. On the other hand, in ASBV-infected avocado trees, there was up to a 10,000-fold variation in

ASBV concentrations in different trees (12).

Palms from areas with different incidences of cadang-cadang disease were found to contain comparable levels of CCCV, indicating that there was no relationship between levels of CCCV in palms and disease incidence in a particular area. For example, similar amounts of CCCV were detected in leaf samples from diseased palms in areas where there were only a few isolated cadang-cadang infected trees in the late stage of disease, and in areas where the disease incidence was 20% with palms in all stages of disease. In the former, there was no active spread of disease, while in the latter a considerable number of new infections appear every year. This indicates that spread of disease is not dependent on the amount of viroid present in infected palms. Symptom expression is also not a function of viroid concentration since there were no significant differences between levels of cadang-cadang viroid in early stage palms with mild leaf symptoms and medium and late stage diseased palms with severe symptoms.

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