ACIAR WORKING PAPER
NO. 51 AUGUST 1998

Report on ACIAR-Funded Research on Viroids and Viruses of Coconut Palm and other Tropical Monocotyledons 1985–1993

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Australian Centre for International Agricultural Research
Canberra

ISBN 1 86320 219 6

FOREWORD

Cocos nucifera is a unique tropical plant. In its domesticated form it depends on humans for its distribution, selection and improvement. It produces food, fibre and shelter almost continuously when water is adequate, and will survive periods of drought. Most coconut palms are grown in small-holder rural communities, where they provide not only an immediate food source, but also cash in the form of copra.

The Philippines is one of the most successful exploiters of coconut products for local use and export income. Coconut development in the Philippines is a remarkable story, with a complex interplay of management, research and development at the national and international level. These factors have kept the crop modernised and relevant to the needs of the purchasers of its products.

Cadang-cadang disease is a major player in the story of coconuts in the Philippines. It has been the reason for prohibition of movement of Philippine coconut germplasm to many countries. The disease was first reported in a plantation on San Miguel, an island close to Tabaco. Recognition of a specific and unique progression of symptoms on palms as the disease advanced through the island was the first chapter in the story of cadang-cadang. Very few people apart from plant pathologists could identify the disease in plantations where other diseases, pests, nutritional deficiencies, typhoons and lightning strikes could also cause low production, yellowing, reduction in crown size or death. The discovery that a viroid (coconut cadang-cadang viroid, or CCCVd) causes cadang-cadang provided a means of diagnosing the disease.

This publication describes many aspects of the work that followed the discovery of the viroid agent of the disease. This phase has included attempts to develop and improve a range of diagnostic procedures suitable for varied applications, to look for resistance, to describe the distribution and epidemiology of the viroid, and to search for a vector. Aspects such as variation in the viroid molecule and variability in disease symptoms under experimental and field conditions have also been studied. The work has centred on the Albay Research Center of the Philippine Coconut Authority, where a research team of scientists have undertaken a broad-based program for nearly 20 years. International support was initially provided by the Food and Agriculture Organization of the United Nations and, for the period 1984 to 1993, by ACIAR. At the suggestion of reviewers of the ACIAR program we have assembled this publication as a collection of reports by the scientists who carried out the projects. You may find occasional examples of apparent repetition of methods, but we have not attempted to amalgamate reports because we were aware that the methods differ in outline and purpose, and we wanted the reader to be able to view each contribution in its own context. We consider that this provides an insight into the way in which knowledge, laboratory technology and the demands of answering practical questions on the distribution of the viroid, risk of loss and control have been combined for a common purpose. Because the work on cadang-cadang was the first study of any 'virus-like' disease of coconut palm, we have also included chapters in which we describe our involvement with newly discovered viruses that infect palms, and one chapter on a disease of unknown aetiology. In this way, we hope that this publication will provide an entry point for subsequent research on viroids and virus-like diseases of coconut palm. Our observations during the survey described in section B lead us to believe that a number of virus-like pathogens await discovery.

This publication illustrates one of the emerging problems in plant pathology, where the application of new sensitive molecular techniques can show that a pathogen or a molecule related to it are more widely distributed than the apparent disease. Observers are entitled to ask, 'Where is the disease?' The scientist who is confronted with this situation must consider a number of possibilities:

- Genetic and environmentally-induced variability in the performance of trees may mask poor performance due to a pathogen.
- Losses may be occurring even in the absence of apparent disease.
- The disease may be presymptomatic and may yet develop.
- The agent may be a mild variant and could mutate to a severe form.
- The environment may be modifying the overt syndrome of the disease but it could change to allow expression.
- Interactions could occur with extant or new pathogens to enhance the risk of new diseases appearing, or of productivity being reduced.

It can be seen from these reports that there are examples where CCCVd can be found in the absence of the disease, that it mutates to a severe form, and that viruses of palms are known that could interact with cadang-cadang if the separate pathogens were able to combine through uncontrolled movement of infected material. Lack of knowledge of the mode of spread of the viroid remains a major deficiency in our knowledge of the disease cycle. The mode of spread is another risk factor that needs to be considered by plant pathologists concerned that the epidemic of cadang-cadang not be allowed to repeat itself in other countries. There are insufficient data to recommend control measures for cadang-cadang. The only way to eliminate the risk of new outbreaks occurring still appears to be to prevent the movement of infected plant material.

Although coconut can still be considered to be a 'Cinderella' crop, its cousin, the African oil palm (*Elaeis guineensis*), is enjoying a remarkable expansion phase. The detection of an oil-palm-associated viroid with sequence similarity to CCCVd, and preliminary indications that it may spread both vertically from the parents representing the narrow genetic base of this crop, and horizontally within plantations, supports the need to find out what these CCCVd related sequences are doing. Given the immense financial and environmental cost in setting up new commercial enterprises, investors and scientists need to agree that germplasm is the best available and that it can perform to its full genetic potential. Diagnostic methods are now in place to test germplasm for the presence of viroid-related sequences; it should now be relatively simple to ensure that high-quality oil palm germplasm is free of such sequences, and so avoid the risk of effects that they may cause.

The report that other monocotyledonous species in many countries contain CCCVd-related sequences is intriguing. Determination of their sequence relationships to the CCCVd may provide an insight into the origins and evolution of viroids in monocotyledons.

We believe that this report provides a valuable insight into some of the efforts that have been made to develop a knowledge of the viroid and virus pathology of the hitherto largely ignored coconut palm. We present it on behalf of the authors in the hope that it may be useful in determining the future direction of research.

The editors

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ACKNOWLEDGMENTS

We gratefully acknowledge the support and cooperation of all countries, institutions and individuals involved with the work described in this book, especially the following. (Addresses and affiliations at the time of collaboration are given; some may no longer be current.)

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1 CADANG-CADANG DISEASE OF COCONUT PALM— AN OVERVIEW

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Introduction

The name 'cadang-cadang' is derived from 'gadan-gadan', which in the Bicol dialect of the Philippines means dead or dying (Rillo and Rillo 1981). The name itself is non-specific, and has led to confusion in the recognition of the disease in the past. However, it now refers to a premature decline and death of coconut palms in the Philippines first recognised early this century, associated with viroid infection. The synonym 'yellow mottle decline' is not in common use. 'Tinangaja' is the name used for a similar disease in Guam, also associated with a viroid.

The earliest unsubstantiated report of cadang-cadang was in the Bicol region of Luzon Island, in Camarines Sur province, in 1914 (De Leon and Bigornia 1953; Kent 1953; Velasco 1961). A disease with the symptoms now known to be specific for cadang-cadang was first described on San Miguel Island in Albay Province in 1931 (Ocfemia 1931); its incidence was reported to be 25% in some areas of the plantation. The disease subsequently destroyed most of the plantation.

The early difficulty of distinguishing the viroid-induced cadang-cadang from other disorders or diseases is illustrated by a report that a nutritional form of cadang-cadang could be controlled by cultural practices (Celino 1946a, b), and the subsequent suggestion that the disease had a physiological form and an infectious form (Celino 1947). Therefore, evaluation of the history of cadang-cadang must take account of the difficulty of recognising the disease on the basis of symptoms alone, and of the relatively small number of people trained to recognise the disease.

Following the observations on San Miguel Island, reports of the disease began to come from sites at increasing distances from the Bicol peninsula (Price 1971) (Fig. 1.1). Water or disease-free coconut-growing areas frequently intervened. These observations suggested that cadang-cadang had spread rapidly and extensively from an initial origin in the Bicol peninsula between 1914 and 1963. This hypothesis was supported by reports that losses of trees increased with time. For example, between 1931 and 1946, disease incidence on San Miguel Island increased from 25% to 90% in some areas (Kent 1953), and fewer than 100 palms survived to the 1970s. Roadside surveys in the Bicol region gave the following estimates for the number of diseased palms: 1951: 1 788 000; 1952: 4 569 000; 1953: 5 527 000 (Kent 1953); and 1957: 7 927 000, when crop losses of $16 million were estimated (Price 1971).

Disease incidence ranged from 9% to 61% in different provinces, and more than 12 million palms were estimated to have been killed by cadang-cadang between 1926 and 1971. The number of new cases of disease estimated to have occurred in 1978 was 391 000 and in 1980 was 209 000 (Zelazny and Pacumbaba 1982a). This represents a decline in incidence since

1960. By about 1980, more than 30 million palms are estimated to have been killed by the disease since it was first recognised (Zelazny et al. 1982).

Speculation on the cause of the disease has been rife. International support for research on cadang-cadang began about 1950 (Reinking 1950). The importance of the disease became more widely recognised, and the disease syndrome was described in some detail (Price 1957; Nagaraj et al. 1965). The Food and Agriculture Organization of the United Nations supported collaboration between a local and a foreign laboratory so as to introduce techniques and facilities not available locally. This led to an active decade of research (1973–1983), which showed that cadang-cadang was a viroid disease and allowed the introduction of specific diagnostic tests. Concurrent intensive ecological studies were introduced with the objective of determining the mode of natural spread and developing a model of disease epidemiology (Rillo and Rillo 1981).

Continued research on cadang-cadang runs parallel to the coconut improvement program in the Philippines, which is based on the production and evaluation of inter-population F1 hybrids. To minimise the risk of an epidemic occurring in new plantings based on these lines, attempts are being made to find plants that are resistant or tolerant to cadang-cadang.

Distribution and Economic Importance

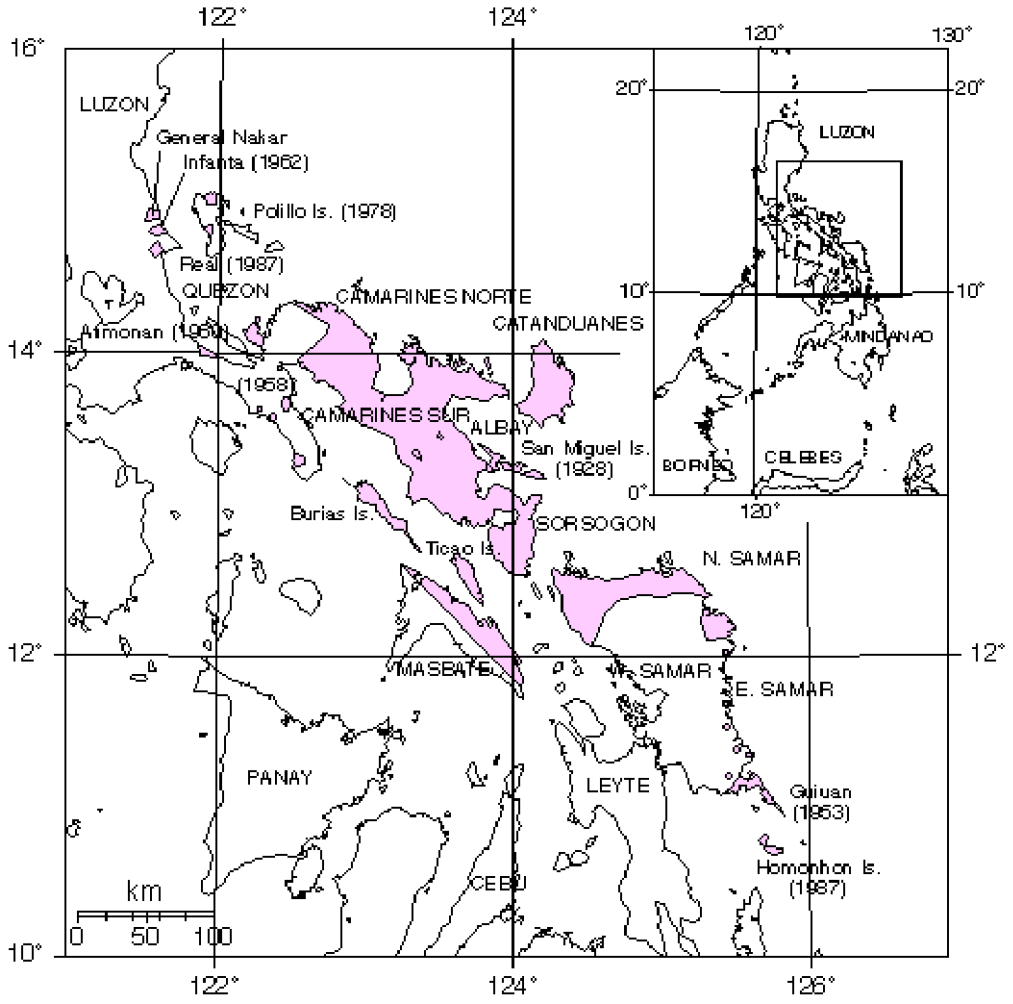
Cadang-cadang is widely distributed on the Bicol Peninsula, Masbate, Catanduanes, Northern Samar, and smaller islands in this zone (see Chapter 5). Outlying outbreaks have been found at and around Infanta (Lat. 14°40'), and in Eastern Samar south to Guiuan, including some adjacent small islands. Small isolated groups of diseased palms have been found north of the main boundary at Atimonan, but surveys of the area west of this to Batangas province, and north to Laguna, have found no evidence of the disease (see Chapter 5). No cadang-cadang has been reported from plantations or experimental plantings in Southern Mindanao.

The disease continues to spread. A rapid increase in incidence was reported between 1984 and 1986 in the provinces of Sorsogon (29% new cases a year), Camarines Sur (11%), Camarines Norte (7%) and Catanduanes (51%), whereas no or minor increases were reported elsewhere. Surveys of boundaries of incidence indicate that cadang-cadang spreads at about 0.5 km a year, and thus the distribution is relatively stable.

Economic losses arise from the cessation of nut production on diseased palms an average of 5 years before they die from the disease. If palms are not replaced until they die, and if it is assumed that replacement takes 5–8 years to reach full bearing, 10–13 years of production may be lost from each diseased site. Assuming an average nut production of 80 per palm per year, and a copra yield of 250 g per nut, the total loss per site would be 200–260 kg of copra. At a price of US $0.40 per kg, the loss per infected site would be $80–$104 (the cost of employing a labourer for 2–3 months). This loss could be considerably reduced if diseased palms were replaced when symptoms first appeared. Nevertheless, if we assume a cost of $100 per site, the 391 000 estimated new cases in 1978 and 209 000 in 1980 could have cost $39 million and $21 million respectively.

Tinangaja disease (Boccardo 1985) was first reported as a destructive disease of coconut palms on Guam in 1917. The disease destroyed the coconut industry of Guam over the next 40 years,

Figure 1.1. **Distribution (shaded areas) and year of first recognition of cadang-cadang disease at several sites in the central Philippines. (For current disease distribution see Chapter 5, Fig. 5.1)**



apparently spreading slowly. The incidence was nearly 100% in some areas, whereas others were almost free of disease. No commercial coconut industry has existed on Guam since 1946 but the disease is still widespread on the island.

Limited surveys using a polyacrylamide gel electrophoresis (PAGE) assay failed to detect CCCVd in coconut palms with foliar decay disease in Vanuatu, or with Kerala wilt or tatipaka disease in India (Randles 1985). A limited survey of the North Marianas, Yap and Palau in 1983 failed to detect CCCVd in coconut palms (B. Zelazny and J. W. Randles, unpublished data).

However, in 1987, a viroid-like RNA similar to CCCVd was detected in Solomon Islands in oil palm with yellow leaf spotting, and also in some coconut palms. The incidence of oil palms with the yellow leaf-spotting symptom was low (ca 1%). This was the first report of a CCCVd-like RNA in plantation-grown oil palm, and the first report that a CCCVd-like RNA occurred outside the Philippines–Guam area. The coconut palms with the suspected viroid did not show symptoms typical of cadang-cadang. An ACIAR-funded project was initiated to characterise

the viroid-like RNA and survey its incidence in the Solomons and other regions of the South Pacific (Hanold and Randles 1991b).

Symptoms of Cadang-Cadang Disease

Palms infected with CCCVd progress through a well defined series of changes culminating in death (Fig. 1.2). Recognition of these stages is important for field diagnosis, and consequently the more obvious morphological changes have been best described. For convenience, diseased palms in the field are classed as being at early (E), mid or medium (M), or late (L) stage (Randles et al. 1977). The early stage has recently been subdivided because major changes in disease and viroid development occur at this stage (Imperial et al. 1981; Mohamed et al. 1982).

Figure 1.2. **Early (E), medium (M) and late (L) stages of cadang-cadang disease.**

The stages are as follows:

E_0: Viroid detected in the youngest fronds; palm symptomless.

E_1: 1– 2 years after E_0, newly developing nuts become more rounded and have equatorial scarification; no leaf symptoms.

E_2: More nuts are rounded and scarified; chlorotic leaf spots appear; inflorescences are stunted, with tip necrosis and loss of some male florets.

E_3: Leaf spots enlarge; fewer nuts are produced; new inflorescences are stunted and sterile.

M: Spathe, inflorescence, and nut production decline and then cease; leaf spots are more numerous.

L: Fronds decline in size and number, leaflets become brittle; leaf spots coalesce, giving a general chlorosis; crown size is reduced and the palm dies.

This progression of symptoms is remarkably constant in the Philippines, with some variation in intensity. The E stage lasts an average of about 2 years in 19–30-year-old palms but lasts up to 4 years in older palms. The M stage lasts for an average of just over 2 years. The L stage to death averages about 5 years. Direct estimates give the mean duration of disease to death as 9 years, whereas indirect estimates give a duration of 7.5 years for 22-year-old palms, and 15.9 years for 44-year-old palms (Zelazny and Niven 1980). The rare infected palms in the field that show symptoms before they begin bearing do not bear nuts, even though they survive well beyond the age of bearing.

Although root deterioration has been reported, studies of histological changes have concentrated on the leaf. Rillo et al. (1972) reported that leaflets from infected palms were thinner, and that palisade and mesophyll tissue were disorganised. This hypoplasia suggests that the disease induces changes at the cell differentiation phase of leaf development. The yellow spots on expanded leaves do not develop from a point of necrosis. Light microscopy shows that chloroplasts are distinguishable in the yellow area but are paler than in the adjacent green tissue. Electron microscopy of the green–yellow interface showed that chloroplasts were vesiculate, starch was accumulated, lamellae were disorganised, and some tannin body accumulation occurred in vacuoles (Randles 1985).

It is noteworthy that the yellow spots (sometimes described as water-soaked spots) do not appear until fronds have reached the third or fourth position in the crown; that is, 3–4 months after they first expand. Exposure to sunlight is thought to be important in the development of spots, as shaded areas show less intense spotting. Thus, the degeneration of chloroplasts and the production of yellow zones may be a later phase initiated by either light or heat, or it may be due to changes in leaf physiology as the leaf changes from an immature, parasitic, white state to a green source of photosynthates. Rasa (1968) reported phloem necrosis in the apical meristem of diseased palms, but Rillo et al. (1972) saw neither phloem degeneration nor proliferation.

Inoculated palms show similar leaf spot development, reduced frond production, reduced crown size and slower growth. Production of inflorescences, or nuts, in palms inoculated as seedlings is rare. Tinangaja differs from cadang-cadang in that nuts are characteristically small and elongated, and lack a kernel (Boccardo et al. 1981).

Only members of the Arecaceae have been successfully inoculated with CCCVd; for example, *Areca catechu* (betel nut palm), *Corypha elata* (buri palm), *Adonidia merrillii* (manila palm), *Elaeis guineensis* (African oil palm), *Chrysalidocarpus lutescens* (palmera), *Oreodoxa (Roystonea) regia* (royal palm), *Ptycosperma macarthuri* (Macarthur palm), and *Phoenix dactylifera* (date palm) (Imperial et al. 1985). Most of those infected were stunted, and all showed yellow leaf spotting. Naturally infected African oil palms at the Albay Research Center became sterile and died (Randles et al. 1980b). So far, no inoculated plants in 44 species from 12 other families have become infected.

The Viroid Agent

CCCVd was first detected in diseased coconut palms by fractionation of nucleic acids isolated from the leaves (Randles 1975b). Two small disease-associated RNAs were identified, the

smaller of which was slightly larger than tRNA. They were shown to have a number of physical properties similar to those described for potato spindle tuber viroid (PSTVd) (Randles et al. 1976). Subsequent isolation, purification (Table 1.1) and inoculation showed that these molecules were infectious (Randles et al. 1977; Mohamed et al. 1985). Several years after successful inoculation, palms showed yellow leaf spotting, reduced growth and reduced frond production (Fig. 1.3), and generally failed to produce inflorescences at the time when normal palms had begun bearing. Parallel studies with tetracycline injection of diseased palms failed to implicate mycoplasmas or other prokaryotes in cadang-cadang (Randles et al. 1977). Long-term observations of inoculated palms have confirmed that they retain symptoms typical of cadang-cadang. It has yet to be shown that inoculation of bearing palms with viroid reproduces the symptoms induced by natural infection in the field, such as necrosis of inflorescences and cessation of nut production. Nevertheless, Koch's postulates have been essentially satisfied in that an agent has been isolated, purified and inoculated to produce a disease virtually identical to that with which it was first associated.

Figure 1.3. **Artificially inoculated seedling (left), 6 years after inoculation, showing stunting, sterility and disordered pinnae, compared with a healthy seedling.**

Table 1.1. Preparation of purified CCCVd from coconut leaf.

1. Blend chopped leaf tissue in at least 4 volumes of 0.1 M Na_2SO_3 (w/v).
2. Extract juice; clarify by low-speed centrifugation.
3. Add PEG 6000 to 8%, dissolve, and incubate at 4° for 1–2 h.
4. Collect precipitate by centrifugation at 10 000 *g* for 15 min.

5. Extract nucleic acids from precipitate by adding equal volumes of 1% sodium dodecyl sulphate and 80% phenol, emulsifying for 30 min, and re-extracting the aqueous phase with phenol:chloroform (1:1). Precipitate nucleic acids from aqueous phase with 75% ethanol in 0.1 M sodium acetate.

6. Fractionate on polyacrylamide gels, first with non-denaturing, then denaturing (8 M urea) conditions.

7. Elute CCCVd in 0.5 M ammonium acetate, 0.001 M EDTA, 0.1% SDS.

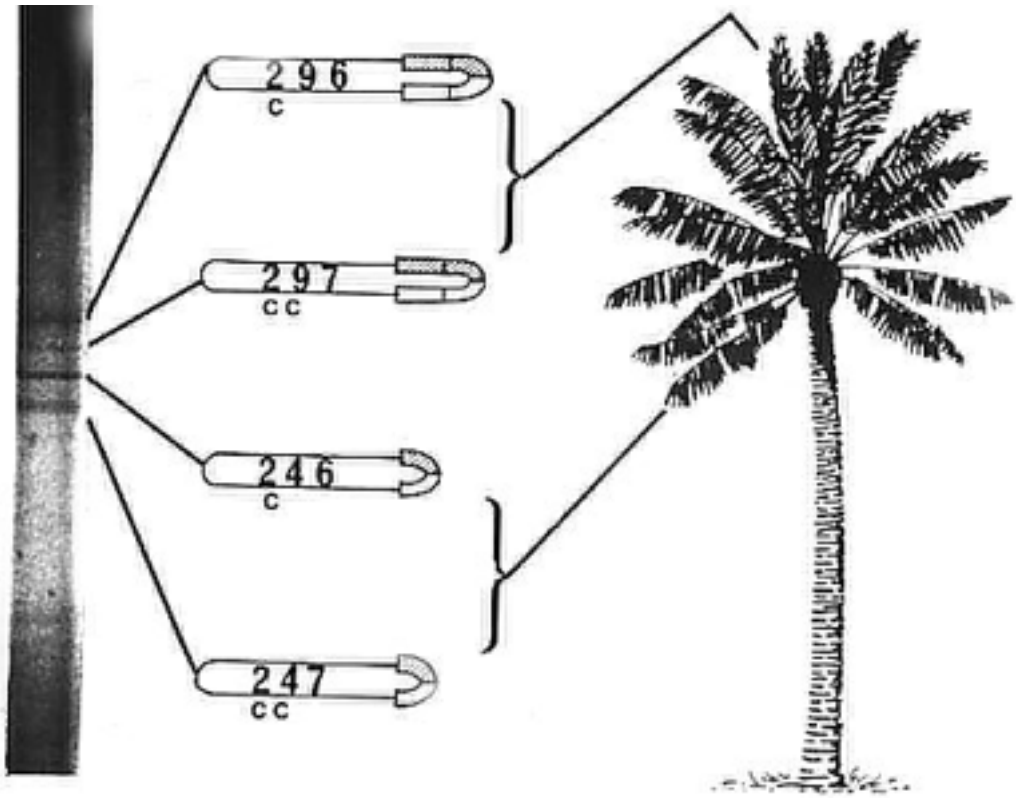
The two disease-associated RNAs, which were shown to be monomeric and dimeric forms of CCCVd, are isolated simultaneously from infected palms. Both are naked, single-stranded, circular (Randles and Hatta 1979) and infectious (Mohamed et al. 1985). Both adopt a rod shaped, partially double-stranded form under native conditions, owing to internal base pairing, and can be denatured to an open circle by heating (Randles et al. 1982). Linear forms of both are isolated with the circular forms, and are separated from them by electrophoresis under denaturing conditions. The linear forms are the same size as the circular forms, and are also infectious. Other molecular variants are shown in Fig. 1.4.

In infected tissue, CCCVd appears not to be closely associated with cell organelles or membranes. Whereas several other viroids sediment with tissue debris, and PSTVd appears to be associated with nucleoli (Riesner 1987b), CCCVd is not sedimented from sap extracts at centrifugal forces sufficient to sediment particles down to 130S (Randles et al. 1976). Nevertheless, the association of CCCVd with material precipitated from tissue extracts with either polyethylene glycol (PEG) or ammonium sulfate indicates that CCCVd is associated with some cell component. Whatever is associated with CCCVd is small, because the 'complex' recovered by PEG precipitation has a sedimentation coefficient below about 40S, and it appears to be tenacious, because CCCVd can be resuspended and precipitated with PEG through at least 3 cycles. Its composition is unknown, but it is removed by phenol-SDS deproteinisation, and the purified viroid is not precipitated with PEG. A study of its nature may provide evidence of the cellular site of accumulation of CCCVd.

The sequences of CCCVd (Haseloff et al. 1982) and tinangaja viroid (CTiVd) (Keese et al. 1988) have been described (Fig. 1.5). The basic form of CCCVd is a 246-nucleotide molecule. It contains a central conserved region of about 44 nucleotides that is common to most viroids. In its presumed native state this sequence would form a structure in the central part of the molecule, with a partially double-stranded region comprising 13 base pairs and small single-stranded loops. Unlike other viroids, CCCVd has a number of molecular forms, which vary at the right-hand end of the molecule (Fig. 1.4).

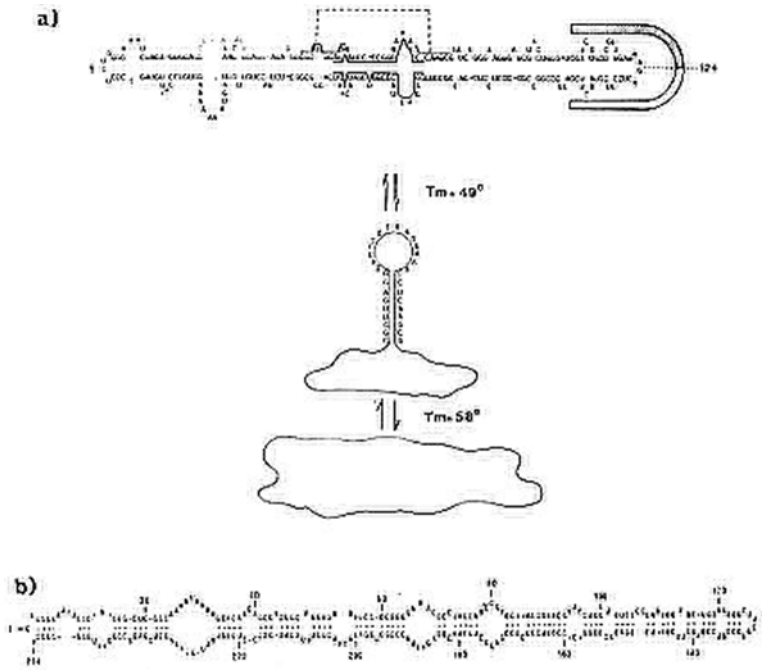
This variation is due to the reiteration of either 41, 50 or 55 nucleotides, producing larger forms of the viroid of between 287 and 301 nucleotides. A minor variation also occurs at position 197, where an additional cytosine may be inserted to produce a basic form comprising 247 nucleotides, and the corresponding large forms. The additional cytosine appears to induce a significant change in the native structure of CCCVd, as the 246- and 247 nucleotide variants can be separated by PAGE under non-denaturing conditions but not under denaturing conditions (Imperial and Rodriguez 1983).

Figure 1.4. **Separation of the four forms of the CCCVd monomer on a 20% non denaturing polyacrylamide gel (left). The small forms (246 and 247) appear first in inoculated palms but are replaced in newly developing fronds by large forms with a reiterated right terminus (296 and 297).**



CTiVd has 254 nucleotides, and has 64% overall sequence homology with CCCVd 246. This variation in sequence could account for both the cross-hybridisation between CCCVd and CTiVd, and the difference in symptom expression of cadang-cadang and tinangaja diseases. CTiVd has not been shown to have the larger molecular forms described for CCCVd, but it does have a dimeric form (Boccardo et al. 1981; Keese et al. 1988).

Figure 1.5. **Primary sequences of CCCVd (a) and CTiVd (b), showing probable base paired and single-stranded regions of the native molecule. For CCCVd, the right-hand end enclosed by the horseshoe bracket is reiterated to produce the large form of the viroid. The central conserved region is marked with a bold line inside. Complementary sequences each side of the conserved region (connected by the dashed line) are able to base-pair just above the melting temperature of 49°C to produce an intermediate in which the small, single-stranded hairpin loop contains part of the central conserved sequence. This intermediate melts above 58°C. For a map of molecular domains see Chapter 15, Fig. 15.1.**



Classification of CCCVd

A classification system for viroids is still evolving.

Viroids can be separated into six groups on the basis of their nucleotide sequence. The groups are represented by PSTVd, CCCVd, hop stunt viroid (HSVd), apple scar skin viroid (ASSVd), avocado sunblotch viroid (ASBVd) and hop latent viroid (HLVd). The sequence similarity between the groups ranges from 39% to 60%, whereas within the groups homology ranges from 65% to 100% (Puchta et al. 1988). CCCVd and CTiVd fall into the same group, and CCCVd differs from the other viroids on the basis of sequence homology, its size, which is the smallest of all the viroids, and biological properties such as its relatively narrow host range and systematic progression of molecular forms.

A comparison of viroids on the basis of the sequence of their central conserved region places them in three groups, named the ASBVd group, ASSVd group, and the PSTVd group (Koltunow and Rezaian 1989). CCCVd is placed in the PSTVd group.

Elena et al. (1991) placed viroids in four subgroups with the following type members: PSTVd (pospi viroids), CCCVd (cocad viroids), HSVd, and *Coleus blumei* 1 viroid. A consensus phylogenetic tree produced by Hernandez et al. (1992) places CCCVd closest to PSTVd.

Disease Cycle

The molecular form of CCCVd isolated from palms is related to the stage of disease development. The small 246/247 form is the first to be detected in young fronds of presymptomatic palms and is the only form detected in all subsequently emerging fronds for about 2 years, until symptoms develop in the nuts (stages E_0 and E_1). As leaf symptoms appear on the newly developing fronds (E_2), progressively larger amounts of the large 287/296/301 nucleotide form, and decreasing amounts of the small form, are isolated. Both forms are detected in fronds for approximately one year, but as these fronds mature and move down through the canopy they are replaced by fronds containing the large form only; this persists through stages M and L. The viroid is not translocated to older fronds, and specific forms appear to persist in a particular frond (Mohamed et al. 1982).

A systematic but more subtle progression has been observed for the forms containing either one cytosine (e.g. 246 or 296) or two cytosine (e.g. 247 or 297) residues at nucleotide position 197. If form 246 appears first in a palm, the transition is 246, 247, 296, 297. If 247 is the first detected, only the 297 form is found later in infection (Imperial and Rodriguez 1983). All forms are infectious (Table 1.2). In the field, the frequency of occurrence of CCCVd 246 in early stage palms at 4 sites was greater than that of CCCVd 247 (PCA 1986).

Table 1.2 **Infectivity of the four molecular forms of CCCVd as determined by assay for viroid at 6, 12 and 24 months. Means with the same letter are not significantly different at P = 0.05.**

CCCVd inoculum	Percentage infection at		
	6 months	12 months	24 months
247	18a	44a	49
246	21a	45a	46
297	4b	19b	40
296	1b	11b	35

The nature of viroid replication and pathogenesis is not understood, but one could speculate that the increase in the size of CCCVd with disease progress may be related to the development of more severe symptoms. Conversely, however, the molecular changes may be a consequence of cell metabolic changes that in turn cause faulty viroid replication.

The observation that inoculation with the large CCCVd leads to the detection of the large CCCVd in some infected seedlings, whereas inoculation with small CCCVd led to the detection of only small CCCVd in the seedlings (Mohamed et al. 1985) shows that under some conditions the large form can be present at the early stages of infection. This system would be useful to further test the role of the two forms of the viroid in pathogenesis.

CCCVd, like other viroids such as PSTVd, can be considered as a dynamic structure (Riesner et al. 1979). It is rod-like in its native state (Riesner et al. 1982), but as it is heated in vitro it undergoes thermal denaturation through an intermediate structure until it finally melts. It shows a first thermal transition at about 49°C, in 10 mM NaCl, and appears to be converted to an intermediate structure that exposes 14 nucleotides of the central conserved region in a small hairpin loop, subtended by a stem of 9 base pairs and a large single-stranded loop (Randles et al. 1982). The nucleotides of the stem are complementary sequences either side of the conserved region. Further heating to about 58°C melts this stem to produce a fully denatured circle. It has

been suggested that viroids could cause disease by interference with, or the regulation of, exon splicing at the level of eukaryotic mRNA processing (Dinter-Gottlieb 1987). The exposure of nucleotides in the central conserved region that have homology with introns and small nuclear RNA could suggest that such a structure, if it occurred in vivo, may be involved in pathogenesis.

Inoculation Methods and Disease Rating

Inoculation is used for testing infectivity, resistance screening, and studies of disease development. Inoculum is prepared by extracting nucleic acids from the PEG-precipitated component of early-stage diseased coconut palms (containing CCCVd 246 or 247). SDS, phenol and chloroform extraction is generally sufficient, but the inoculum may be purified further by treatment with 2 M LiCl (to precipitate high-molecular-weight, single-stranded RNA) or 1% cetyltrimethyl ammonium bromide (CTAB) (a specific precipitant for nucleic acids) (Ralph and Bellamy 1964). A number of trials have been done to optimise inoculation efficiency. Inoculum is delivered in 0.1 mL amounts by hand-primed, high-pressure injection to coconut sprouts (i.e. emerging seedlings). The infectivity of the inoculum is not markedly affected by factors such as the degree of purification of the viroid, the amount of viroid injected into each sprout in the range 0.8–8 μg, or the buffer used to dissolve the inoculum (0.067 M phosphate, pH 7; 2 mM Na_2EDTA, pH 6.0; 100 mM sodium acetate, pH 7.6; or 150 mM NaCl/ 15 mM sodium citrate, pH 7.9). Inoculum is infectious for at least 2.5 years when stored as an ethanol precipitate at –15°C. Predarkening of seedlings for 72 hours enhances susceptibility. Injection of 0–5-day-old sprouts of partially dehusked nuts gives 40%–60% transmission after 1 year and 70%–95% within 2 years. This is higher than that for 1–3-month-old seedlings. Inoculation of sprouts exposed by complete dehusking leads to high mortality.

Table 1.3 shows the relative rates of infection with high-pressure injection to either the outside of the sprout or the exposed inner part of the base of the sprout, by pricking with a needle through drops of inoculum into the base of the sprout, or by mechanical inoculation by rubbing sprouts with inoculum in carborundum. Highest efficiency and lowest mortality are achieved by high pressure inoculation to the outside of the sprouts. Two injections are as effective as four, when the same total dose is given. Injection of roots or the tips of leaflets is inefficient compared with injection at the bases at the centres of leaflets. High-pressure injection of the spear leaf of mature palms is being tested as a means of inoculation for established palms.

Table 1.3. Transmission of CCCVd by a range of inoculation methods. Mean of 4 trials, each with 5 test plants. Means with the same letter are not significantly different at P = 0.05.

Method of inoculation	Percentage of test plants surviving after inoculation	
	Total	Infected
High-pressure injection, outer	64a	48a
High-pressure injection, inner	29b	26b
Pricking	7b	7bc
Mechanical	13b	0c

The end-point of infectivity has been estimated to be between approximately 200 pg and 20 ng of viroid injected (PCA 1986). After injection, sprouted nuts are left under partial shade in elevated beds for about 4 months, then transplanted to polybags in the field for a 2–3-year observation period.

Replicated groups of 5–15 seedlings are used in infectivity assays, and rates of infection are determined by PAGE assay for viroid at 0.5, 1 and 2 years after inoculation. No data are available on the mode of natural inoculation in the field. No vector has been found. Current work is directed towards determining whether palms may be infected by harvesting bunches of nuts with scythes, or by pollination with pollen from diseased palms at stage E. CCCVd has been detected in peduncles, husks of young nuts, and anther extracts, so that accidental cutting of palms with a contaminated scythe, or movement of anther components by insects, are possible routes of spread that need further investigation.

Screening for resistance in individuals within populations of breeding lines is done by inoculation as described above. Rating is based on the cumulative percentage infection, as determined at intervals of 0.5 to 1.5 years for up to 5 years. No breeding lines have so far been found to be immune to inoculation by injection. Further work will be directed towards selecting individual seedlings within populations that 'avoid' infection after repeated inoculation treatments. These will then be grown to maturity and self-pollinated, and their progeny will be screened as above. Field resistance is being assessed simultaneously by exposing selected breeding lines within areas of high incidence.

The assay of infection depends on analysis for CCCVd by PAGE or molecular hybridisation. The large scale of trials has required the development of sensitive, rapid and simple diagnostic procedures (Table 1.4).

Table 1.4. Analysis of leaf samples for CCCVd (Randles et al. 1992a).

1. Place 0.5–2 g leaf samples in a plastic bag, with 1–2 vol. of 100 mM Tris·HCl, pH 7.2, 100 mM Na acetate, 10 mM EDTA and 0.5% thioglycerol.
2. Crush with a hammer, squeeze juice into a centrifuge tube, add 0.5 vol. 90% phenol and 0.5 vol. chloroform and mix.
3. Separate aqueous phase by low-speed centrifugation; precipitate nucleic acids with 75% ethanol.
4. Collect precipitate and assay by PAGE or molecular hybridisation.

Epidemiology

Surveys of cadang-cadang incidence and distribution have been done for many years to provide information on distribution, rates of increase or decline, and rates of spread. A major objective has been to determine whether these patterns matched the behaviour of potential vectors. Early disease surveys were done in strips along roads by trained observers recording disease incidence, and in plantations, where incidence and spread was mapped. It was concluded (Sill et al. 1964; Price and Bigornia 1971, 1972) that disease incidence was related to the age of the plantation. Incidence was negligible before plantations were 10 years old, but after this, a linear regression of incidence on age was observed, at least until incidence reached 50%–60%. Evidence supporting the view that cadang-cadang spread from palm to palm was presented by Price and Bigornia (1972), in conflict with a previous hypothesis that other species were the source (Holmes 1961). The reliability of the conclusions drawn from these studies is limited by their restricted scope, a result of inadequate research resources.

Nevertheless, the following conclusions were justified at the time.

- Cadang-cadang is contagious and is not likely to be due to soil, physical, biological or chemical factors as had been suggested previously by Velasco (1961, 1982).
- It has a scattered, apparently random distribution, the rate of spread is slow, and if a vector is responsible for spread it is either rare, inefficient, or sluggish.

One immediate benefit of this early work was that replanting was shown to be a feasible means of maintaining yields in infected ageing plantations, because young palms had no significantly greater risk of becoming infected when growing next to diseased palms than when grown in new plantations in the same area.

More intensive studies since 1975 (Zelazny et al. 1982; Zelazny 1979, 1980; Zelazny and Pacumbaba 1982a) have confirmed some of the earlier conclusions while giving a more comprehensive picture of rates and type of spread in a number of different situations. Zelazny and his coworkers have concluded from their observations that the area within which cadang cadang occurs has increased very little in the last 26 years. Surveys at the boundaries of disease distribution showed that outward spread was less than 0.5 km a year. New infections can be found up to several hundred metres ahead of a boundary. Where isolated pockets of infection were known to exist, they appear not to have expanded markedly since 1960. Thus, there is no evidence that cadang-cadang originated at one point in the Philippines.

These conclusions provide an alternative hypothesis to that which arose from the sequential identification of cadang-cadang at increasing distances from the site of first recognition. These observations implied that the disease had spread rapidly outwards from a single source in the last 70–80 years. The alternative hypothesis states that cadang-cadang is endemic in the Philippines and that some factor is responsible for its present pattern of distribution. A number of factors could be involved, such as mutation of CCCVd to a pathogenic form, protection of some palms because of genotype or ‘mild strain protection’, distribution of a putative vector or reservoir, or palm age distribution, but none of these has been implicated so far.

There is no doubt that epidemics of the disease occur, but observations at a number of sites indicate that they have occurred at different times in different places. Thus, an epidemic was observed in the Albay province in 1951–57 but the incidence is now declining. In contrast, parts of the neighbouring Camarines Sur province are now experiencing 50%–70% incidence of disease, whereas in the same area in 1956 the incidence was below 3% (Zelazny et al. 1982).

Within the boundaries of disease distribution (Chapter 5, Fig. 5.1), cadang-cadang incidence is highly variable. In small areas, diseased palms are not clustered, but over large areas, centres of high and low incidence are seen. Zelazny (1980) surveyed an area of 1492 km^2 and attempted to correlate disease incidence with variables such as site, altitude, abundance of different vegetation, palm density, palm age, rainfall and soil conditions. A significant negative correlation was observed between altitude and incidence and, in agreement with earlier observations, a positive correlation was observed between palm age and incidence. Three beetle species (*Oryctes rhinoceros, Plesispa reichei*, and *Hemipeplus* sp.) were more abundant in areas with high disease incidence (Zelazny and Pacumbaba 1982b), but so far no insects have been shown to transmit cadang-cadang.

Patterns of disease increase vary from site to site. Some fit a 'simple interest' pattern, others a 'compound interest' pattern. The lack of a specific pattern of disease increase does not allow the source of infection in plantations to be inferred. The low rates of spread mean that trials to determine mode of transmission must be very large.

An evaluation of the epidemiology of cadang-cadang by surveying for symptoms is of limited value because of the imprecise incubation period between inoculation and symptom appearance, and the difficulty of recognising early symptoms. Rapid and portable diagnostic tests based on PAGE (Table 1.4) have been developed for monitoring the movement of the viroid in the field and in experimental plots. A number of very important questions have to be answered before epidemiology is sufficiently well understood for control measures to be developed. For example, does spread occur from coconut palm to coconut palm, or from another host species to coconut? What is the rate, range and pattern of viroid movement from infector plants? Is there periodicity of spread? Long-term trials have been set up to help answer some of these questions (see Chapter 5).

Possible Strategies for Management

A major objective of research on cadang-cadang has been to obtain sufficient information on disease aetiology, epidemiology and host range to allow control measures to be developed. The nature of the coconut industry also requires that control measures be simple, reliable and cost-efficient.

No control measures can be recommended at present, but several possible avenues for future development can be considered.

Replanting

The replacement of infected palms, or replanting of infected plantations, appears to be the earliest recommended means of reducing the losses due to cadang-cadang (Bigornia 1977). This recommendation was based on the observation that the rate of spread in new plantings was not influenced by the proximity of infected palms. Although having an economic cost in lost production and cost of planting, this practice has allowed production to continue in the cadang-cadang area.

Eradication

Control by removal of diseased palms has been attempted in two sets of trials. In 1952–55, diseased palms were removed every 3 months; 2 years later, the rate of spread was reported to be about 1/10 that in the untreated area (Zelazny et al. 1982). A trial that began in 1979 on an isolated island with about 300 000 palms, in which all palms with disease symptoms were cut annually, showed that there was a marked decline in new cases of disease during the first two years, but in the third year, as many new cases occurred as at the beginning of the trial. This trial is continuing, but it is noteworthy that early attempts to eradicate cadang-cadang from apparently new outbreak sites have not succeeded—infected palms still appear in these areas.

Although at first observation eradication appears to be ineffective for controlling cadang cadang, two points must be considered. First, the period from inoculation to symptom appearance in mature coconut palms is not known, although it is known that from the first detection of CCCVd in fronds, 1–2 years may elapse before symptoms are first seen (Mohamed

et al. 1982). Second, as shown in Table 1.2, CCCVd is detectable earlier in palms inoculated with CCCVd 246 or 247 than in palms inoculated with CCCVd 296 or 297. This suggests that the smaller forms, which are present in the presymptomatic E_0 and E_1 stages, may be more rapidly transmitted than the larger. Control by eradication of all palms with viroid detectable by molecular methods should therefore be tested.

Resistance

Resistance and tolerance are currently being sought in field populations that have been exposed to cadang-cadang for many years (Bigornia and Infante 1965) and by the inoculation of specific seed lines (Imperial 1980). The development of field resistance relies on selecting and breeding from survivor palms in high-incidence areas. Preliminary results suggest that disease incidence is lower in selected plants, and that they may also be less readily infected by inoculation. The current hypothesis that cadang-cadang has been present in the Bicol region for several centuries suggests that much of the selection for field resistance may have already occurred in populations established in the region. Nevertheless, several generations of selection are required to ensure that apparent survivors are not merely escapes, and that development of field resistance is a practical goal.

The current interest in the heterosis obtained with dwarf × tall hybrids has meant that parent material of unknown susceptibility to cadang-cadang is being used to produce F1 seed. Inoculation of CCCVd is now used routinely to seek resistance or tolerance in these parent lines for future breeding programs. Moreover, these lines are being tested by exposure to natural infection in high incidence areas.

Vector control

Although the search for a vector has been unsuccessful, the probability that one or more exists means that control measures may eventually be directed towards vectors.

Mild strain protection

Mild strains of viroids may cross-protect against severe strains (Horst 1975; Niblett et al. 1978), at least in the short term. The lack of other control strategies could favour the use of mild strain protection. The mild strain of PSTVd differs only slightly in its nucleotide sequence from the severe strain (Gross et al. 1981), and it seems likely that eventually the sites on viroid molecules where changes in sequence can be induced so as to modify pathogenicity will be defined (Keese and Symons 1985). Research on the forms of CCCVd has shown that considerable variation at the right end of the molecule apparently does not affect their ability to infect. Variation at the left end of the molecule has not been reported by Haseloff et al. (1982), but Rodriguez and Randles (1993) have described mutations in the pathogenicity and central conserved domain of CCCVd that are associated with the severe 'brooming' form of the disease (see Chapter 13). Experiments need to be done to determine the stability and pathogenicity of mutants. Evidence from other viroids has shown that mutation is frequent, and that passage through different plant species can be associated with selection of viroid mutants (Fagoaga et al. 1995).

At present, little is known about the natural occurrence of mild strains of CCCVd. CTiVd shows too much difference in sequence from CCCVd to allow regions of molecular variation to be defined that may be responsible for the particular symptoms of each disease. The detection of CCCVd-like sequences in coconut and oil palms in Solomon Islands (Hanold and Randles 1991b) should allow research to begin on the relationship between sequence and pathogenicity,

and initiate the collection of a range of naturally occurring variants for evaluation in mild strain protection. Preliminary results that have shown that ginger (Zingiberaceae) and arrowroot (Marantaceae) collected in the Philippines contain sequences partially homologous with CCCVd (Rodriguez 1993) suggest that other naturally occurring variants of CCCVd will be found as molecular hybridisation is more widely used in surveys for the distribution of CCCVd.

Conclusions

Cadang-cadang is the most important of the viroid diseases, principally because of its lethality and the losses it continues to cause in the Philippines (Maramorosch 1993). The threat that it may present to other countries is shown by the identification of CCCVd-like molecules in oil palm and coconut palm in a number of other countries (Hanold and Randles 1991b). An epidemic continues in the Philippines; the apparent absence of epidemics in other countries may be due to several factors that need further investigation. For example, the putative vector of CCCVd may not be active, the variant of CCCVd may be less severe than CCCVd in the Philippines, the palms may be tolerant to infection, or symptom expression may be influenced by the environment. Work is in progress to improve the sensitivity, rapidity, simplicity and portability of diagnostic procedures for CCCVd for use in the field. Such tests are essential to evaluating the epidemiology of CCCVd, and to proceeding in the quest for the mode of spread.

2 DIAGNOSTIC METHODS APPLICABLE TO VIROIDS

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Introduction

Viroids are the smallest known pathogens and have been found only in plants. Unlike viruses, they do not have a protein coat, and consist solely of a small, circular, single-stranded, infectious RNA molecule that can both replicate in the host cell and be transmitted independently of any other microorganism. Viroids range in size from 246 to approximately 375 bases (Keese and Symons 1987) and have strong, internal nucleotide sequence homologies leading to substantial base pairing, which gives them a rod-like shape in their native state. They have a characteristic melting pattern with a transitional intermediate owing to sequences in the conserved region (Riesner 1987a) (see Chapter 1, Fig. 1.5). Most viroids known so far have been found in cultivated plants and are transmitted mechanically by human cultural practices. Some can spread naturally through insects, seed and pollen (Diener 1987a), or by still unknown means (coconut cadang-cadang viroid (CCCVd), coconut tinangaja viroid (CTiVd)) (Hanold and Randles 1991a).

Little is known about the physiology of pathogenicity and the replicative mechanisms of these pathogens, except that they appear to require DNA-dependent RNA polymerase II for replication, and they have no DNA intermediates (Sänger 1987). They have been classified according to the presence of certain nucleotide sequences (see Chapter 1).

With viroids thus defined by their molecular characteristics, diagnosis of a suspected pathogen as a member of this group must therefore rest on proof that a viroid-like molecule is present and responsible for the disease under investigation. Four important points should be considered:

- First, most viroids, such as CCCVd and CTiVd in palms, appear to be present in their hosts in very low concentrations. Also, the nature of the host tissue may make it difficult to extract the viroid RNA (e.g. palms, grapevine). It is therefore essential to first develop an extraction method that is efficient and suitable for a potential viroid and that has also been optimised or modified for each different host species.
- Second, single-stranded RNA is very susceptible to degradation by ribonuclease, and operations such as collection, storage of material and sample preparation need to minimise the risk of enzymatic degradation of viroid. Appropriate controls, including infected and healthy plant tissue, are necessary to check the efficiency of extraction and the safety of storage conditions.
- Third, because plant extracts contain many small host nucleic acids, components suspected of being viroids must be shown to have the characteristic properties of viroids—that is, single-stranded, circular RNA, in the size range 246 to 380 nucleotides.
- Fourth, even if one or more viroids have been detected, association with disease needs to be established, for example by appropriate inoculation studies. This is because several ‘latent’ viroids have been found accidentally that do not cause symptoms on some or all host plants (Desjardins 1987; Diener 1987b; Hanold and Randles 1991b; Puchta et al. 1988), and even closely related strains can vary in pathogenicity.

This chapter describes general methods that are useful as the first steps for investigating diseases possibly caused by viroids. Specific cases may require special modifications to achieve best results. Such modifications are included in later chapters where applicable; where there are differences, descriptions given there take precedence over this chapter.

Methods

Preparation of samples

Collection and storage of material
Choose a range of young, but fully expanded, undamaged leaves with well developed symptoms (if present). Seal them in plastic to prevent drying out, keep them cool, and process them immediately or store them at –20°C. Discard brown, wilted or bruised tissue, since this indicates that cell breakdown has occurred with likely degradation of the RNA.

Viroid purification
To limit contamination by RNAses, general sterile working practices are adopted and gloves are worn at all times. Treat all solutions, glassware, and other materials contacting nucleic acid preparations by autoclaving, by baking for 2 h at 180°C, or with 2 M KOH in 90% ethanol. The work area should be cool, clean and free of draughts and dust, which might carry RNAse contamination.

An example of a protocol successfully used for the extraction of CCCVd, CTiVd and related viroids from palms and herbaceous monocotyledons is given in Table 2.1. Dicotyledon tissue may allow simplified procedures. Antioxidants ($SO_3$2-, βmercaptoethanol, monothioglycerol) should be included in the extraction buffer to prevent oxidation of plant polyphenols to tannins, with consequent browning. Work should proceed as quickly as practicable to minimise degradation by plant RNAses released during cell disruption. For CCCVd-related molecules, the addition of polyvinyl polypyrrolidone (PVPP) has proved advantageous during extraction, although its specific role is not known. Note, however, that some viroids might stick to PVPP or bentonite (which is sometimes used to adsorb RNAses) and thus can be lost. Only CCCVd and CTiVd are known to be precipitated from crude extracts with polyethylene glycol (PEG, mol. wt 6000); deproteinised viroids will not precipitate with PEG (Randles et al. 1976). Efficient inhibitors of RNAse are phenol, 0.1–1% SDS, and human placental RNAse inhibitor (RNasin) in the presence of at least 1 mM dithiothreitol (DTT), but the inhibition is reversible and RNAse activity will be restored once these inhibitors are removed.

Table 2.1. An extraction procedure for CCCVd-like RNA from palms and other monocots (Hanold and Randles 1991b). Compare with Table 1.1, Chapter 1, which describes a simpler method to be used only for coconut leaf tissue.

1. Chop 10–20 g of leaf and blend in 120 mL of 100 mM Na_2SO_3.
2. Strain through muslin and shake for 30 min at 4°C with 20 g/L polyvinyl polypyrrolidone (PVPP).
3. Mix vigorously for 5 min with 50 mL of chloroform; centrifuge at 10 000 *g* for 10 min.
4. Add polyethylene glycol (PEG) 6000 at 80 g/L to the aqueous supernatant.
5. Precipitate for 2 h at 4°C; spin at 10 000 *g* for 10 min to collect the pellet.

6. Dissolve the pellet in 2 mL of 10 g/L sodium dodecyl sulphate (SDS).

7. Add 2 mL of aqueous phenol (900 g/L) containing 1 g/L 8 hydroxyquinoline; shake vigorously for 1 h.

8. Collect aqueous supernatant by centrifugation; re-extract with 1 mL of phenol and 1 mL of chloroform for 5 min; spin at 10 000 *g* for 10 min.

9. Adjust NaCl in supernatant to 0.1 M, add cetyl trimethyl ammonium bromide (CTAB) to 3.3 g/L, and allow precipitation to occur for 30 min at 0°C.

10. Centrifuge for at least 30 min at 10 000 *g* and wash pellet 3 times with 0.1 M Na acetate in 75% ethanol.

11. Air dry pellet and resuspend as needed.

Other steps may be used in the purification of viroids. Treatment at 0°C for 16 h with 2 M LiCl can selectively precipitate high-molecular-weight nucleic acids from the viroid extract. Extracts in TE (10 mM Tris·HCl pH 7.5, 1 mM EDTA) buffer can be either purified by adsorption of viroid to DE52 cellulose columns and elution with 1 M NaCl in TE, or fractionated on Qiagen (J. W. Randles, pers. comm.) columns (Diagen GmbH, Düsseldorf, Germany) and elution of viroid-sized nucleic acids with 0.4–0.7 M NaCl. Viroids are precipitated by adding 0.8 volumes of isopropanol or 3 volumes of ethanol and sedimented by centrifugation for at least 30 min at 10 000 *g*. Precipitation with cetyl trimethyl ammonium bromide (CTAB) is efficient at removing polysaccharides and other contaminants and is suitable for CCCVd-like RNA, but its reliability needs to be checked for new viroids. Precipitate with 3.3 g/L CTAB in 0.1 M NaCl on ice for 30 min, centrifuge for 30 min at 10 000 g, wash the pellet three times by dispersion in 0.1 M Na acetate in 75% ethanol and resediment. Density gradient centrifugation has been useful in obtaining pure viroid (e.g. PSTVd) when large quantities of material are available.

A widely used method for purification is preparative gel electrophoresis, usually with a combination of non-denaturing and denaturing gels, as described below.

Short extraction methods can sometimes be devised for routine screening of one particular host species after a viroid has been characterised. For example, Table 1.4 in Chapter 1 shows a short method for CCCVd screening in coconut in the Philippines. Such methods need to be evaluated with different viroids or host species before adoption.

Gel electrophoresis

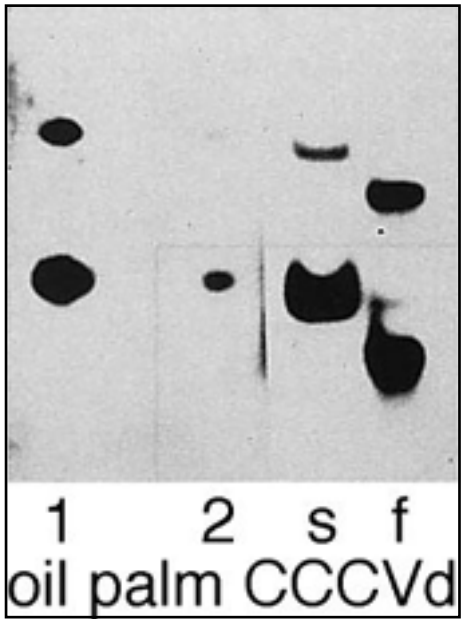
Viroids migrate in most gel systems with a mobility less than that expected for their molecular weight. Common, single-stranded, linear RNA size markers are therefore not suitable for estimation of viroid molecular weight, and interpretation of gel patterns is often difficult even in comparison with known viroid markers. If linear viroid forms are present, their size can be estimated in denaturing gels by comparison with linear RNA markers. Because only a small percentage of total plant-cell nucleic acids is represented by the infecting viroid, partially purified viroid preparations are usually required for analysis by gel electrophoresis.

Polyacrylamide

Polyacrylamide gel electrophoresis (PAGE) is invaluable for viroid diagnosis.

Choice of gel matrix. By varying the polyacrylamide concentration in the range 5% to 20%, with the ratio of acrylamide monomer to bisacrylamide dimer ranging from 97:3 to 99.6:0.4, pore size and gel strength can be varied to suit particular applications. A 20% gel with a 97:3 monomer:dimer ratio in a TBE (Sambrook et al. 1989) buffer system (90 mM Tris, 90 mM boric acid, 3 mM EDTA) gives high resolution and sharp bands. This system has sufficient precision to separate mutants of CCCVd (see Chapter 1, Fig. 1.4), which differ in their secondary structure as a result of single nucleotide deletions or additions (Rodriguez 1993), but it is also very susceptible to distortion from impurities in the sample and therefore requires relatively pure preparations. In contrast, for example in 5% PAGE with a 98.8:1.2 monomer:dimer ratio, CCCVd forms differing in size by only 1 nucleotide are not resolved, but the sample tracks are not distorted by low levels of impurities (Fig. 2.1).

Figure 2.1. **CCCVd and extracts of oil palm with 'genetic' orange spotting syndrome (GOS) analysed by 5% PAGE and Northern blot hybridisation assay using full-length CCCVd-cRNA probe (Hanold and Randles 1991a). Left: 1, 2 = GOS oil palm extracts containing viroid-like molecules related to CCCVd; right: s = slow (296/7 nucleotides) and f = fast (246/7) forms of CCCVd, both showing monomers (lower major bands) and dimers (upper minor bands).**



Denaturing PAGE. Addition of 8 M urea or formamide to a polyacrylamide gel running under high temperature conditions, or changing the pH of the buffer, gives it 'denaturing' properties, so that internal H-bonding between base pairs in nucleic acids is removed and secondary structure is lost. Viroids in such systems denature from their native rod-like state to open circles and migrate more slowly than their linear forms. Complete denaturation is achieved by running gels at temperatures of about 50°C in the presence of 8 M urea. Diagnostic tests for viroids have often been based on PAGE under both non denaturing and denaturing conditions, and on the characteristic change of behaviour of these molecules when subjected to both sets of conditions.

Consecutive PAGE. Samples are analysed on a non-denaturing gel. Bands under investigation are stained with either ethidium bromide or toluidine blue (without fixing) and cut out or, in the absence of visible bands, the region expected to contain possible viroids is cut out according to appropriate markers. The gel slices are then loaded on top of a second, denaturing gel containing 8 M urea of either identical or different gel strength (Schumacher et al. 1986). Viroid bands,

owing to their open circular configuration, should migrate more slowly than the now single-stranded, linear nucleic acids that were in the same position in the first non denaturing gel when the viroid had its rod-like native configuration.

Bidirectional PAGE. This method uses the same principle as consecutive PAGE, except that the gel is not cut. Instead, after the first electrophoresis, the current is reversed and nucleic acids are denatured in the original gel by increasing either temperature or pH (Schumacher et al. 1986). Since gel strength cannot be varied, good separation of viroid bands and linear nucleic acids is more difficult. Unless used by experienced investigators, this method also often fails to denature all nucleic acids completely for the return 'denaturing' run and thus may not give a clear result. Small amounts of viroid can also remain trapped in a large excess of linear nucleic acids and thus escape detection.

Two-dimensional PAGE. In this system, a whole track from a non-denaturing gel is cut out and placed across a gel containing 8 M urea and then electrophoresed at a 90° angle to its first run (Schumacher et al. 1983). This has the advantage that the complete molecular range can be screened for possible viroids, rather than having to cut out a certain area. This is especially advantageous when looking for unique nucleic acids associated with an unknown pathogen. Gel strengths can be varied to get good separation in both dimensions. It is also possible to reverse the sequence of conditions—that is, denaturing gel as the first dimension and non-denaturing as the second. This may result in less background if samples are not completely clean, and it will make staining of the second gel with ethidium bromide possible for preparative applications, since the native molecules will stain much more strongly with this method than denatured viroids. Running the second gel at a 90° angle prevents trapping of small amounts of viroid by excess linear nucleic acids since viroid molecules do not have to pass through a gel area contaminated with other molecular species.

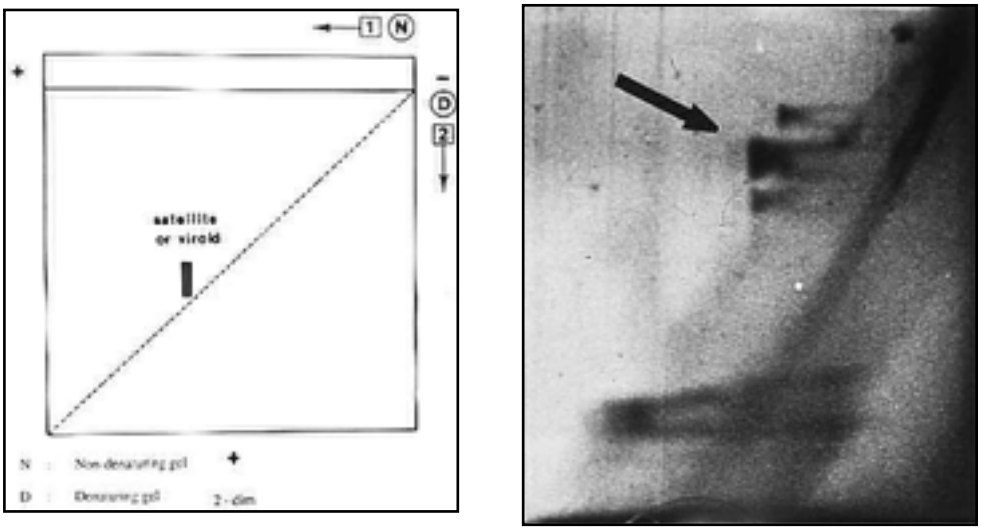
If used in connection with a sensitive detection method (silver stain or molecular hybridisation; see below), the two-dimensional PAGE system is a sensitive and definitive test for the presence of small, circular nucleic acids in a preparation, and is thus a powerful tool in the search for viroids (Fig. 2.2).

Gradient electrophoresis. PAGE with temperature, urea or pH gradients can be used to visualise the specific melting pattern characteristic of viroids (Riesner 1987a).

A purified preparation is applied across a gel containing a gradient and electrophoresed in a direction perpendicular to the gradient. Characteristic molecular transitions occur at certain points of the gradient. These are visualised after staining by a curve representing the altered electrophoretic mobilities of the native or partially melted molecular forms with sharp changes occurring at the respective melting points of distinct molecular structures.

To determine the degree of relationship between viroids or strains, heteroduplex molecules can be formed in vitro between the species under investigation by liquid hybridisation. These can then be electrophoresed with the current parallel to a gradient (Wartell et al. 1990). Heteroduplexes will denature when they reach a specific point in a gradient, depending on their degree of homology, and will be slowed down there, resulting in different band positions even for molecules with only one base change.

Figure 2.2. **Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). (Left:) Principle of detection of circular nucleic acids. (Right:) An example of a 5% non-denaturing (right to left)/denaturing (top to bottom), 2-D PAGE showing different molecular forms of circular CCCVd separated from the diagonal representing linear RNAs (monomeric forms indicated with the arrow; dimeric forms to the right of the monomers near the corner of the gel). Stained with silver nitrate.**



Agarose

Agarose gel elecrophoresis (AGE) on gels between approximately 1.2% and 2% can be used to analyse extracts, with either a TBE or a TAE (Sambrook et al. 1989) buffer system, followed by staining with ethidium bromide. These systems are not too much affected by impurities still present in the preparations, and are therefore suitable for screening partially purified samples. However, ethidium bromide staining is not as sensitive as the silver stain used for polyacrylamide gels, and bands are not as sharp as in PAGE. This results in a tendency for weak viroid bands to disappear in background fluorescence. Ethidium bromide stains denatured viroid molecules less efficiently than the native RNA; that is, at about 1/10 the sensitivity obtained for the native, partially base-paired form. Prestaining CCCVd with ethidium bromide before AGE affects its electrophoretic mobility (Randles, pers. comm.). Agarose gels, with or without sodium dodecyl sulphate (SDS), can be useful for preparative purposes.

Staining methods

Silver. The most sensitive stain for polyacrylamide gels is silver (Imperial et al. 1985). In theory, detection of as little as 50 pg of viroid should be possible. In practice, however, a heavily staining background in the sample tracks often interferes with detection of bands. Depending on the host tissue, this background may be almost impossible to remove, even after extensive purification (e.g. oil palm tissue extracted for CCCVd). With such materials, the alternatives are ethidium bromide staining (see below) or blotting of gels with subsequent molecular hybridisation assay (see below).

The presence of bands derived from host cells is another frequently encountered problem. If for a particular host–viroid combination such bands are present in the viroid region, identification of the real viroid bands may be very difficult, even more so for a new viroid that is not yet available in purified form as a marker or probe.

Nucleic acids stained with silver are permanently fixed in the gel. This stain can therefore not be used in the first phase of consecutive or 2-dimensional PAGE or for preparative gels.

Ethidium bromide is an intercalating fluorescent dye that is reversibly bound to nucleic acids and can be used in all gel systems. In non-denaturing gels it is 90%–98% less sensitive than silver stain. It causes fewer problems with background staining, but denatured viroid molecules are inefficiently stained; ethidium bromide cannot be recommended for these. It is ideal for preparative gels, since it can be incorporated in the gel during electrophoresis, thus minimising risk of breakdown of viroid due to extensive manipulation of the gel. As ethidium bromide is strongly mutagenic and a suspected carcinogen, take appropriate safety precautions to avoid any skin contact.

Toluidine blue staining is at least 90% less sensitive than ethidium bromide, except for denatured viroid molecules, where it provides about the same sensitivity of detection. It can be used in conjunction with acetic acid to reversibly fix nucleic acids in the gel, or as an aqueous stain for preparative gels. Since the procedure requires an extensive time for destaining, the aqueous stain usually results in partly diffused bands, and the risk of RNA breakdown in the gel must be considered.

Sequential staining of gels is possible, but only in the order ethidium bromide – toluidine blue – silver stain.

Viroid recovery from gel pieces

If electrophoresis is used for preparative purposes, all equipment should be RNAse-free. Great care must be taken not to contaminate the preparations with RNAses during recovery from the gel.

RNA can be eluted from gel pieces by incubation at 37°C in 10–20 volumes of NH_4 acetate/ SDS/EDTA (Maxam and Gilbert 1980) overnight, removal of the liquid, addition of the same amount of fresh buffer, incubation for another 1–2 h, and ethanol precipitation of the viroid from the combined eluates. The presence of SDS will effectively inhibit RNAses. Recovery is best from agarose, and is progressively less efficient from PAGE with increasing gel concentration, but is generally less than approximately 70%.

Alternatively, gel pieces can be inserted into dialysis tubes filled with 1–2 mL of electrophoresis buffer containing a few μg of carrier nucleic acid, RNasin and 1 mM DTT, and electroeluted for 1–2 h. Reversal of the current for 1 min at the end of the run frees RNA adhering to the tubing. Remove the buffer, rinse the bag, and precipitate with ethanol. Recovery should be similar for all gel systems and somewhat higher than by the first method. The major cause of loss of RNA is adhesion to the dialysis membrane. The risk of RNAse contamination may be slightly higher than by the diffusion method described above owing to more extensive handling of materials. DE52 cellulose (Whatman anion exchanger) can be used to concentrate RNA from large volumes (Sambrook et al. 1989).

Nucleic acids can also be extracted from gel pieces by centrifugation (Heery et al. 1990). Inside a larger centrifuge tube (e.g. a 1.5 mL Eppendorf tube), place a shorter one (e.g. a 0.5 mL Eppendorf tube) with a rim diameter exceeding that of the inside of the large tube and with a fine hole at its lowest point. Place siliconised glass wool, then the gel slice (agarose or

polyacrylamide), in the upper tube and centrifuge at 10 000 g for 10–15 min to remove the aqueous phase, including nucleic acid, from the gel piece. Discard the top tube with the gel, vacuum dry the liquid obtained from the gel piece or add salt and ethanol to precipitate the viroid (depending on amount present and the required purity).

Viroid purified in low-melting-point agarose can be recovered by melting and phenol extraction (Sambrook et al. 1989). However, partial denaturation of the RNA can occur during the melting of the agarose.

Electron microscopy
Electron microscopy of samples spread under denaturing conditions can be used to identify circular viroid molecules and estimate their size (Randles et al. 1987) (Fig. 2.3). It cannot be used for diagnostic purposes on tissue sections or crude extracts, since the small viroid rods and circles cannot be positively identified when mixed with other nucleic acids.

Tests for nucleic acid characteristics

Distinction between DNA and RNA
The following tests can be used to distinguish between RNA and DNA. Treatments are applied before assay by PAGE.

Alkali. RNA is degraded by incubation in 0.5 M KOH at room temperature for 30–60 min. DNA is not affected (Randles et al. 1986).

RNAse. Selectively degrade RNA with DNAse-free RNAse. To prepare DNAse-free RNAse, dissolve RNAse A in 10 mM Tris (pH 7.5) plus 15 mM NaCl at 10 mg/mL, heat to 100°C for 15 min and allow to cool slowly. Use at 10 μg/mL in TE buffer, incubating at room temperature or 37° for 1 h (Randles et al. 1986).

DNAse. Selective degradation of DNA by DNAse. Use RNAse-free DNAse in the appropriate buffer containing 10 mM $MgCl_2$, preferably with RNAsein and DTT (Sambrook et al. 1989).

Distinction between single- and double-strandedness
Enzymatic. S1 nuclease digests only single-stranded nucleic acids. RNAse A selectively degrades single-stranded RNA under high salt conditions (300 mM NaCl) and both single- and double-stranded RNA under low salt conditions (50 mM NaCl) (Randles et al. 1986).

Acridine orange can be used to stain gels in a similar way to ethidium bromide, but it will show red fluorescence for single strands and green fluorescence for double strands when viewed under UV illumination (McMasters and Carmichael 1977). It has mutagenic and carcinogenic properties similar to ethidium bromide and should be handled with care.

Electrophoretic. Bands on gels representing single-stranded nucleic acids show the same relationships between size and electrophoretic mobility when electrophoresed under both denaturing and non-denaturing conditions. Bands representing double-stranded nucleic acids show a different size-to-electrophoretic-mobility relationship from single-stranded nucleic acids, so that when they are denatured the mobility of the resultant single strands is difficult to predict. Viroids show reversible denaturation, in that they have a native, partially double

stranded, rod-like structure that melts on denaturation but 'snaps' back once denaturing conditions are removed. They may also adopt intermediate, partially denatured, transition states. This partial double-strandedness and strong secondary structure can cause abnormal behaviour compared with 'true' single strands.

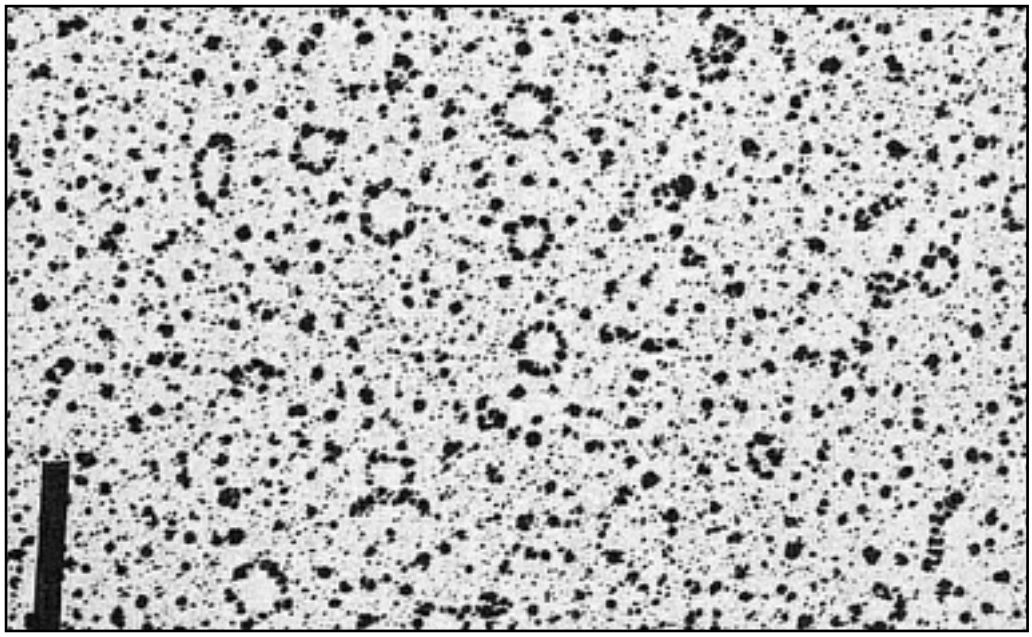
Denaturation by melting internal hydrogen bonding is achieved by boiling the samples with 50% formamide, by glyoxylation (McMasters and Carmichael 1977), or by running samples on denaturing gels (see above).

Distinction between linear and circular nucleic acids

Two-dimensional PAGE (see above) is the method of choice to prove the presence of small circles in a nucleic acid mixture. Bidirectional or consecutive PAGE can also be used.

Electron microscopy of both denaturing and non-denaturing spreads of the purified nucleic acid under investigation (Randles et al. 1987), using standard techniques, can show the presence of rods and circles in the preparation (Fig. 2.3).

Figure 2.3. **Electron microscopy of purified coconut cadang-cadang RNA spread under denaturing conditions, showing circularity of molecules (Randles and Hatta 1979). Grids were stained with uranyl acetate and rotary shadowed. Bar represents 300 nm.**



Molecular hybridisation assays

The following describes aspects of specific concern when working with viroids. General methodology is covered by Arrand (1985) and Sambrook et al. (1989).

Preparation of blots

For both dot blots and gel blots, the positively charged Zeta probe nylon membrane (BioRad) gives excellent results owing to its superior ability to bind and retain small RNA molecules and its ease of handling. It is preferable to uncharged nylon or other kinds of supporting membranes such as nitrocellulose. We routinely prewash the blots for 1 h at 65°C in 0.1 × SSC (20 × is 3 M

NaCl, 0.3 M Na citrate), 0.5% SDS, and include carrier DNA at 1 mg/mL in the prehybridisation/hybridisation mixtures to minimise background signals (Hanold and Randles 1991b) (see hybridisation protocol, Table 2.2).

Both electroblotting and capillary blotting work well for viroids from all types of gels, including even 20% PAGE. Gels are not pretreated for capillary blots (Thomas 1980) and are only briefly equilibrated in transfer buffer for electroblotting to minimise diffusion of bands and loss of the small amounts of viroid in the sample tracks. After transfer, blots are not rinsed, but are immediately air-dried and baked (Hanold and Randles 1991b).

Preparation of probe (Sambrook et al. 1989; Arrand 1985)
Nucleic acid probes suitable for detecting viroid by molecular hybridisation assay can be prepared if at least one of the following is available:

- Purified viroid, suitable for reverse transcription
- Cloned viroid sequences in a suitable plasmid vector (e.g. SP6 system) to make transcripts of cDNA or cRNA
- Specific primers for the polymerase chain reaction (PCR)
- Knowledge of the taxonomic group to which the viroid belongs, so that oligonucleotide sequences in the conserved region may be synthesised for use as probes, or so that probes for related viroids can be used in low-stringency hybridisation
- Knowledge of at least part of the sequence for oligonucleotide probe synthesis.

The most appropriate approach will depend on whether the viroid under investigation is a new one or a variant of a previously known isolate.

cRNA probes are preferable to cDNA probes, because they bind more strongly to the target RNA, giving stronger signals and thus allowing conditions of higher stringency for hybridisation and washing that result in less background and reduce the non-specific binding of the probe. Table 2.2 gives an example of a protocol for detection of CCCVd-like RNA with heterologous CCCVd-cRNA probe.

Table 2.2. An example of a protocol for the detection of CCCVd-related molecules with a heterologous CCCVd-cRNA probe (Hanold and Randles 1991b).

1. Prepare $_{32}$P-cRNA probe by transcription from a full-length CCCVd clone in pSP64 vector and purify by PAGE (Hanold and Randles 1991b).
2. Fractionate nucleic acids by PAGE, equilibrate gels in TAE (9 mM Tris, 4 mM Na acetate, 0.4 mM EDTA, pH 7.4 (Sambrook et al. 1989)) for 15 min, transfer RNA to Zeta probe (BioRad) nylon membrane in TAE at 60 V, 0.6 A for 5 h, and bake membranes for 2 h at 80°C.
3. Prewash blots for 1 h at 67°C in 0.1 × SSC (20 × is 3 M NaCl, 0.3 M Na citrate), 1 g/L SDS, then prehybridise for 15–20 h at 37°C in 0.75 M NaCl; 75 mM Na citrate; 50 mM sodium phosphate at pH 6.5; 5 mM EDTA; 2 g/L each of bovine serum albumin (BSA), Ficoll 400 (Pharmacia), polyvinyl pyrrolidone of mol. wt 40 000, SDS; 1 mg/mL denatured herring testis DNA; 50% deionised formamide.
4. Heat cRNA probe to 80°C for 1 min in 50% formamide, add to hybridisation mix (i.e. 80% prehybridisation buffer containing 110 g/L dextran sulphate) at about 10^6 cpm/mL

final concentration, and hybridise filters at 37°C for 20–40 h in slowly rotating roller bottles.

5. Rinse filters at room temperature in 0.5 × SSC, 1 g/L SDS, then wash for 1 h at 55°C with agitation in 1 × SSC, 1 g/L SDS, for low stringency. Wrap filters in plastic to keep moist; expose at –70°C using intensifying screens for approximately 2 days.

6. Re-wash filters for 2 h in 0.1 × SSC, 1 g/L SDS, with agitation for high stringency. Re-expose for 7–10 days (time may be varied to adjust for expected signal strength).

Since high sensitivity is desirable for diagnosis, and because viroid concentrations are probably low, probes with high specific labelling are needed. None of the non-radioactive labelling methods available gives satisfactory results in our experience, because they show low sensitivity, non-specific binding to plant extracts, or non-specific signals owing to an endogeneous reaction between the sample and substrate, in the absence of conjugated probe. This occurs especially when alkaline phosphatase is used in the detection reaction, for example with a biotin–streptavidin system. Further disadvantages are that consecutive washes at increasing stringencies (see below), multiple probing and variation of exposure times to increase sensitivity are not possible with most non-radioactive probes. Thus, radioactive labelling with ^{32}P is the method of choice in our laboratory. Nick translation and end-labelling do not result in high specific activity of the probe. Our best results by far are obtained with a monomeric ss cRNA probe of high purity from an SP6 or similar RNA polymerase transcription system. We consider the extra effort spent on preparation of this kind of probe worthwhile.

Hybridisation parameters

Stringency of hybridisation can be lowered by using 37°C instead of 42°C, if heterologous viroid isolates are to be detected (Anderson and Young 1985).

Variants of viroids with different levels of homology from the probe can be detected and their homology can be estimated by first washing blots at low stringency, exposing, then re washing at higher stringency, re-exposing, and continuing in this way through several steps of stringency, if desired. Ensure that the membrane is kept moist at all times if further washes are planned, since drying will fix the probe.

The rate of loss of probe compared with a homologous marker preparation can give an estimate of the degree of homology of the strains to the probe.

The probe can be removed by boiling the blots for 20 min in 0.2 × SSC, 0.5% SDS, and the complete removal can be verified by autoradiography if the treatment was carried out within the time limit of activity of the previous probe. The blots can then be re probed following the original protocol. This can be repeated several times when using Zeta Probe nylon membrane without excessive loss of sample.

Amplification and nucleotide sequence determination

To obtain sufficient material for diagnosis, characterisation or determination of a nucleotide sequence, it may be necessary for some viroids to use molecular amplification of the RNA. Amplification may be achieved either by cloning cDNA in bacteria or by in-vitro amplification with PCR (Vos 1987; Puchta and Sänger 1988). Available methodology can be used, taking into consideration the special properties and problems of viroids. Nucleotide sequencing can be

done directly on the purified RNA, or by conventional methods on cDNA (Keese and Symons 1987). However, the strong secondary structure of viroid RNA will require special approaches to overcome compressions on sequencing gels.

Inoculation methods

As a final proof that an isolated viroid is the cause of a disease, Koch's postulates need to be satisfied, and reproduction of the original disease by a purified preparation must be shown.

Many viroids show high infectivity in crude plant extracts. However, for some viroids or particular host species, crude extracts may not contain a high enough viroid concentration for successful inoculation. It is then preferable to use partly purified viroid preparations (e.g. a PEG precipitate in the case of CCCVd (Imperial et al. 1985)). Greater-than-full-length cDNA clones have also been shown to be infectious for some viroids (Sänger 1987).

Inoculation methods can be abrasion, slashing or grafting (Diener 1987), but in the case of CCCVd, for example, only high pressure injection close to the meristem of young seedlings gives sufficiently high rates of infection in coconut (Imperial et al. 1985) (Fig. 2.4).

Figure 2.4. Inoculation of coconut seedling with CCCVd using high-pressure injection Panjet) close to the meristem.

Conclusions

Implication of a viroid pathogen

If a nucleic acid has been implicated as disease-associated, a distinction has to be made between a viral and a viroid pathogen.

To achieve this, the following sequence of tests is suggested:

1. Is the nucleic acid RNA?
2. Does it have a circular form?
3. Is it single-stranded?
4. Is its size in the range of 246–380 nucleotides?
5. Is it infectious when purified?

The properties listed as points 1–5 need to be shown to reveal the viroid nature of a pathogen. Infection may be latent in particular species, and molecular methods are generally necessary to establish host species. For example, HLVd (Puchta et al. 1988), ASBVd (Desjardins 1987), CLVd (Diener 1987) and CCCVd in herbaceous monocots (Hanold and Randles 1991a, b) are latent in some or all known hosts, or symptoms are so mild or non-specific that unequivocal diagnosis by symptoms is difficult. Alternatively, symptoms may take too long to develop to be of practical use (CCCVd in palms: Imperial et al. 1985).

If steps 1–5 demonstrate the viroid nature of the agent, then two further steps can be undertaken:

1. Is its nucleotide sequence characteristic of viroids, and in which taxonomic group does it fit?
2. Develop a specific, sensitive and rapid diagnostic test.

Classification and identification

When the nucleotide sequence of a new viroid is known, classification can be attempted (see Chapter 1). This leads to the possibility of designing group-specific, viroid-specific, or strain specific probes that can be used at different levels of stringency to establish degrees of relatedness or to look for new group members and strains, and for diagnostic purposes. Even single-base mutations can be detected using probe heteroduplexes in temperature gradient gel electrophoresis (Wartell et al. 1990).

Latency

Viroids are probably much more widespread than we are currently aware (Hanold and Randles 1991b). Besides the ones that have been identified because they are causing serious diseases in crop plants, there is likely to be a large number that do not cause obvious symptoms. However, these so called ‘latent’ viroids may still cause yield reduction or some growth inhibition, and ‘latent’ hosts may also act as reservoirs for a viroid that can cause disease under changed environmental conditions or in other economically relevant species. Apparent freedom from symptoms can by no means be regarded as freedom from viroids (Hanold and Randles 1991a, b).

3 DIAGNOSIS OF CADANG-CADANG BY RAPID POLYACRYLAMIDE GEL ELECTROPHORESIS

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Introduction

Cadang-cadang symptoms are unreliable for detecting infection. Reliable diagnosis is based on identifying the viroid RNA in extracts of coconut palms. This chapter describes a simplified, miniaturised and rapid assay for cadang-cadang diagnosis. It involves three steps. In the first, sap is extracted from coconut leaf tissue and deproteinised. In the second, nucleic acids are recovered by cold ethanol precipitation. In the third, direct detection of the viroid is achieved by fractionation of the nucleic acids using polyacrylamide gel electrophoresis (PAGE) and silver staining. The procedure is sensitive, with a detection end-point of about 600 picograms of viroid. It is reliable and suitable for detecting viroid at the early stage of symptom development.

Development of the Method

Initial studies of cadang-cadang involved the use of prolonged procedures for extracting CCCVd from samples of palm tissue (Randles 1975b; Imperial et al. 1981; Imperial and Rodriguez 1983; Imperial et al. 1985). These methods were reliable but required a controlled laboratory environment and specialised equipment. The maximum number of samples that could be extracted in a day was limited to 40, employing 2–3 workers. The procedure took about 2 days before samples were ready for assay by PAGE.

With the establishment in 1986 of field surveys to determine the boundaries of disease distribution, there was a need for a short, efficient method to identify CCCVd and confirm the diagnosis of cadang-cadang disease. The assay system needed to handle many samples, to be suitable for use in the field laboratory, and to be as sensitive as the normal laboratory method.

Tissue and buffer selection

Initial experiments were done to determine which part of the palm was the best for field diagnosis. Primary root tips and leaves were compared because these tissues are easily sampled and readily crushed for nucleic acid extraction. A summary of the experiments in Table 3.1 shows that leaf tissue was better for assay than roots. In some of the methods tested, CCCVd was almost always detected in the leaves, even when corresponding root samples yielded negative results. There were differences between extraction buffers: the phosphate/ EDTA/ mercaptoethanol buffer (PEM) gave the highest number of positives (Table 3.1).

For routine assays, coconut leaves were collected from the third-youngest fronds of palms. Each sample included 1–2 leaflets from the lower, middle and tip positions. Midrib-free leaf lamina (1 g) was used for the total nucleic acid extraction.

Table 3.1. Comparative trials using various extraction methods for CCCVd.

Extraction buffer	No. samples with CCCVd/total no. samples assayed				
	Leaves			Roots	
	Trial A 25 Sep 1986	Trial B 11 Sep 1986	Trial C Nov 1986	Trial A 25 Sep 1986	Trial B 11 Sep 1986
PEM	6/15	33/38		6/15	8/38
PE	6/15			0/15	
NaOAc	5/15			2/15	
NDDT	0/15			0/15	
SO_3	2/8	11/38		2/15	12/37
Ames	2/8	23/38		1/15	10/38
Routine		23/38	9/27		3/38
Revised PEM			18/27		
Revised PEM + LiCl			13/27		

PEM = 0.1 M Na-K phosphate buffer, pH 7.0, 10 mM EDTA, 0.5% 2-mercaptoethanol
PE = 0.1 M Na-K phosphate buffer, pH 7.0, 10 mM EDTA
NaOAc = 0.1 M, pH 7.6
NDDT = 0.2 M Na^2HPO^4, 10 mM DIECA, 5 mM DTT, 0.2% (v/v) Triton X–100
SO_3 = Na_2SO_3, 0.1 M
Ames = 0.5 M NaOAc, 10 mM $MgCl^2$, 20% ethanol, 3% SDS, pH 6.0
Routine = same as SO_3 but using an electric grinder (long method)
Revised PEM = extraction buffer same as PEM but extracted juice is clarified by centrifugation
Revised PEM + LiCl = same as revised PEM but with an additional purification step using 4 M LiCl as follows: The extracted juice is clarified by centrifugation. Clarified sap is mixed with 4 M LiCl stock to make 2 M then left at 4°C for 15 h. Resulting supernatant after centrifugation is precipitated by cold ethanol.

Nucleic acid extraction

The extraction procedure was miniaturised, simple and rapid (Rodriguez et al. 1989). One-gram samples of leaflets were placed in small (10 × 8 cm), heavy-gauge plastic bags. The fresh tissue was crushed with a pestle with 1 mL of cold PEM (0.1 M Na-K phosphate buffer, pH 7.0, 10 mM EDTA, 0.5% 2-mercaptoethanol). The sap extract was recovered from the bag by squeezing the fibre and forcing the juice to one corner of the bag. Approximately 0.5 mL was transferred to an Eppendorf tube. Protein was removed by thoroughly emulsifying it with 0.5 volumes each of phenol and chloroform for 2 min. After centrifugation at 7000 rpm for 10 min, the upper aqueous layer, containing nucleic acids, was recovered. Nucleic acids from an aliquot of the aqueous phase equivalent to 0.3 g of leaf were precipitated with 3 volumes of cold ethanol and sedimented by high-speed centrifugation (10 000 rpm) for 10 min. Pellets were dried in a vacuum desiccator and resuspended in 25 μL of 10 mM sodium acetate/10% sucrose.

Fractionation by PAGE

Electrophoresis was done under non-denaturing conditions in 20% analytical polyacrylamide slab gels—140 × 120 × 0.75 mm—containing a ratio of 32.3:1 of monomer:dimer (Rodriguez et al. 1989). The Tris–borate–EDTA (TBE) buffer system (Peacock and Dingman 1968) was used.

After gels were prerun for 30 min at 200 V, nucleic acid samples and a standard CCCVd marker were loaded into individual wells of the gel. The samples were allowed to enter the gel for 15 min at 75 V before the voltage was increased to 175 V and the gel was run for 6 h. Mobility

of sample components through the gel matrix depends on molecular size and shape and net electrostatic charge of the molecule. This gel system separates the 4 monomeric variants of the viroid: 246, 247, 296 and 297 nucleotides (Imperial and Rodriguez 1983).

Staining of gels
The viroid bands are detected in the gel by silver stain according to Sammons et al. (1981) and as modified by Imperial et al. (1985). Details of the procedure are given in Chapter 4. This staining technique has been shown to have a threshold of detection of about 600 pg of viroid per sample.

Samples showing bands that co-migrated with those of the CCCVd standard were scored as positive whereas those without bands in the marker region were scored as negative.

Storage of gels
Stained gels can be preserved for future reference in a solution of 60 mM sodium carbonate, 3.0 mM sodium azide and 0.5% acetic acid. The gel is first blotted dry with tissue paper and then sealed in a clear plastic envelope with 1 mL of preservative solution. For prolonged storage, gels are kept at 4°C.

Applications
The PEM – bag extraction – PAGE assay method can detect CCCVd as early as 6 months after mechanical inoculation experiments (Mohamed and Imperial 1983), before symptoms are evident.

Although the technique was developed primarily for field diagnosis in the mobile laboratory, it has also been used routinely in the main laboratory as a tool for studying pollen and seed transmission of the disease, for screening for resistance to CCCVd in inoculated coconut populations, and for insect transmission studies, by monitoring coconut test plants for viroid.

The rapid procedure has several advantages over the long methods. It works well under field conditions with ambient temperatures of around 30°C (compared with the controlled laboratory temperature of approx. 22°C). Essential equipment includes a small balance, a portable table centrifuge capable of speeds to 10 000 rpm, and a PAGE apparatus. In contrast, the long method requires a refrigerated centrifuge and an electric grinder. The PEM method involves a 3-step extraction procedure that makes it possible to process about 80 samples in 4 h, whereas the long method entails numerous steps and about 2 days to process batches of about 40 samples. Speed is enhanced and the risk of contamination between samples is minimised by the use of individual plastic bags for crushing leaflets.

From 1986 to 1992, the PEM method has been used during 20 disease surveys for processing more than 9000 coconut leaf samples (Table 3.2).

Table 3.2. Summary of assay for cadang-cadang boundary determination (see Chapter 5), using the rapid PEM–PAGE assay method, between 1986 and 1992.

Year	Survey site	Total no. samples	Mode of assay	Operator*
1986	Quezon Province	57†	uninstalled mobile laboratory	MJBR
1986	Western Samar	68†	"	LPE
1986	Infanta, Quezon	94†	"	MJBR + ZSB
1987	Eastern Samar	63†	"	MTIN + ZSB
1988	Atimonan, Quezon	534	mobile laboratory	MJBR + MTIN + ZSB
1988	Western Samar	599	"	MTIN
1988	Infanta, Quezon	467	"	MTIN
1989	Atimonan, Quezon	787	"	ZSB
1989	Western Samar	604	"	ZSB
1989	Infanta, Quezon	525	"	ZSB
1989	Southern Tagalog Region	287	"	ZSB
1990	Western Samar	594	"	ZSB
1990	Infanta, Quezon	463	"	ZSB
1990	Atimonan, Quezon	462	main laboratory	ZSB + MTIN
1991	Western Samar	574	"	ZSB + MTIN
1991	Infanta, Quezon	463	"	ZSB + MTIN
1991	Atimonan, Quezon	467	"	ZSB + MTIN
1992	Western Samar	746	"	MBO
1992	Infanta, Quezon	562	"	MBO
1992	Atimonan, Quezon	679	"	MBO

* Operator code:
MJBR = Ma. Judith B. Rodriguez
LPE = Lucy P. Estioko
MTIN = Ma. Teresa I. Namia
ZSB = Zosimo S. Bonaobra III
MBO = Maryjane B. Orolfo
†Midrib-free leaf samples were finely cut into 1 mm-wide pieces before extraction; all the succeeding samples were cut into 5 cm-long pieces before extraction.

4 THE MOBILE DIAGNOSTIC LABORATORY

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Introduction

No appropriate control measures for cadang-cadang can yet be recommended, mainly because its natural mode of transmission is still unknown. The source of inoculum and possible mode of spread, however, might be deduced if adequate and sound data on the epidemiology of the disease could be obtained. Epidemiological surveys done previously have been based on symptomatology (Zelazny 1979; Zelazny and Pacumbaba 1982a). Thus the distribution of viroid in asymptomatic palms, as at the very early stage of the disease, has not been recorded.

This paper describes a mobile laboratory that was built for the rapid, cost-efficient and reliable field diagnosis of cadang-cadang viroid infection in coconut. The viroid assay technique developed for use in the mobile laboratory was essentially as described in Chapter 3. Intensive monitoring of viroid spread and distribution has been possible, especially in remote survey sites. In the absence of specific control measures for cadang-cadang disease, early detection followed by eradication (cutting and burning) of diseased palms can now be tested to determine whether it can minimise the spread of infection.

The Assay Procedure

As described in Chapter 3, the procedures developed for normal laboratory use (Randles 1975b; Imperial et al. 1981; Imperial and Rodriguez 1983; Imperial et al. 1985) were modified by the omission or combination of various steps, the use of alternative extraction buffers, and the replacement of large items of equipment with simple instruments. Miniaturisation was also attempted. The ratio of acrylamide to bisacrylamide in gels was varied to improve the resolution of bands and to reduce the time of electrophoresis. Tris–borate–EDTA electrophoresis buffer (Peacock and Dingman 1968) was used throughout, and gels were stained with silver (Sammons et al. 1981; Imperial et al. 1985).

As coconut palms in the Philippines are of the tall type and generally old, leaf sampling is time-consuming. Although sampling of the roots would be more convenient, CCCVd was detectable only in young primary root tips, which are hard to locate in old palms. Furthermore, CCCVd was sometimes detectable in leaflets of frond 3 but not in the primary root tips of the same palm.

Using the rapid CCCVd extraction method (Figs 4.1, 4.2 and 4.3; Table 4.1), approximately 80 samples could be processed under field conditions within 4 h. By changing the ratio of acrylamide to bisacrylamide from 99:1 to 32.3:1, the duration of the polyacrylamide gel electrophoresis (PAGE) run was shortened from 15 h at 125 V to 6 h at 175 V.

Figure 4.1. **A 1 g leaf sample is placed in a plastic bag and 1 mL of PEM buffer is added.**

Figure 4.2. **The leaf sample is crushed with a pestle, then the juice is squeezed out into a centrifuge tube and clarified by low speed centrifugation. The supernatant is collected and nucleic acid is precipitated for 15–30 min with 75% ethanol.**

Figure 4.3. **A silver-stained gel showing the viroid bands. The gel has been removed from the apparatus, placed in a plastic tray, fixed with 10% trichlo-roacetic acid for 10 min, washed 3–4 times with glass-distilled water, and then equilibrated in 0.19% silver nitrate for 3–60 min. It is then rinsed 3–4 times with degassed distilled water before it is developed in a solution of 1% formaldehyde, 2 mM sodium borohydride plus 250 mM sodium hydrox-ide, and enhanced with 70 mM sodium carbonate.**

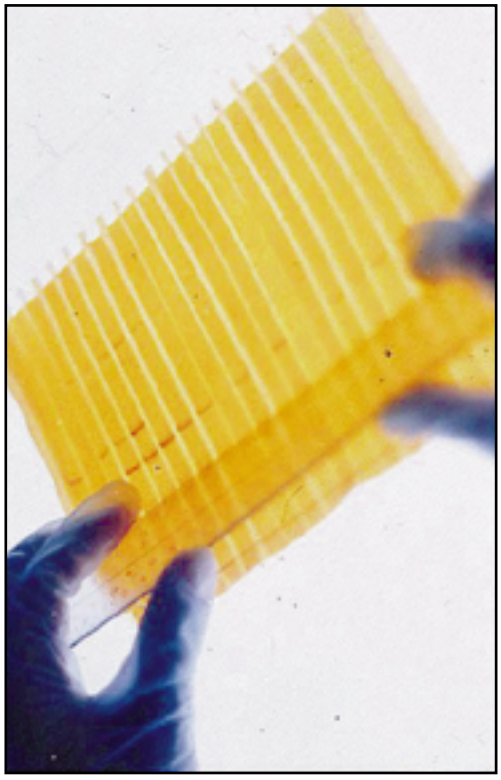

Table 4.1. **Comparison of PEM buffer extraction in the rapid assay for CCCVd with the standard sodium sulphite method (– = not detected; + = detected).**

Sample number	Extraction buffer component SO_3[b]	Extraction buffer PEM[a]
1	–	–
2	–	–
3	–	–
4	–	–
5	–	–
6	+	+
7	+	faint +
8	+	+

a: 0.1 M Na-K phosphate buffer, pH 7.0, 10 mM EDTA, 0.5% 2-mercaptoethanol.
b: 0.1 M Na_2SO_3–PEG method. Grind leaves in 0.1 M Na_2SO_3 and centrifuge. Add PEG to supernatant (to 7.5%), stand sample at 4°C for 2 h, centrifuge, and dissolve recovered pellet in 0.1 M NaOAc. Centrifuge, recover supernatant and precipitate nucleic acids with 3 volumes of ethanol for 15–30 min. Resuspend the nucleic acid pellet in electrophoresis buffer for 20% PAGE assay.

On the whole, the technique worked best with coconut leaf samples, giving a detection end point of about 600 pg of CCCVd. When used to detect CCCVd in other parts of the coconut palm and in other plant species, extracts usually give ambiguous gel patterns.

Economy in chemicals and laboratory equipment was achieved as well: the electrophoresis apparatus, a small balance and a table-top, Eppendorf-type centrifuge were the only essential equipment. The mobile laboratory measured 1.79 × 1.84 × 2.96 m (Figure 4.4). Additional laboratory fixtures were a small fume-hood and a small refrigerator for storing chemicals. A portable generator provided electricity.

Figure 4.4. **Rear view of the mobile laboratory.**

The reliability of the diagnostic laboratory has been tested several times, during determinations of actual disease boundaries in Samar and Quezon provinces. It was also used in establishing epidemiological plots in Atimonan and Infanta, Quezon, and in Santa Margarita, Western Samar.

In a field survey, PAGE assay is preferable to the alternative dot-blot molecular hybridisation assay (MHA) using a radioactive complementary RNA probe. Although the latter is approximately 300 times more sensitive, it requires about 4 days to perform, there is a risk of exposing workers to radioactive materials, and the cost of synthesising and hybridising with the probe is high. However, if such drawbacks can be overcome by using non-radioactive procedures or alternative labels for probes, then the PAGE assay can be replaced.

5 THE EPIDEMIOLOGY OF COCONUT CADANG-CADANG AND DISTRIBUTION OF CCCVd

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Introduction

Coconut cadang-cadang disease was first reported from San Miguel Island, Albay province, Philippines, in 1927 (Ocfemia 1931), and is presently widespread in the Bicol Peninsula, Northern Samar, the southern tip of Eastern Samar and neighbouring smaller islands. During the 1960s, small isolated pockets of diseased palms were reported in Quezon province. It is estimated that up to 30 million palms have been killed by the disease since it was first recognised (Zelazny et al. 1982). The largest losses of coconut palms occurred in 1957, when yield losses of US$16m were estimated (see Chapter 1). Using road surveys and relying on visual observations, 391 000 and 209 000 new cases of disease were estimated to have occurred during 1978 and 1980, respectively (Zelazny and Pacumbaba 1982), representing a decline in incidence since 1957.

Studies of the spread and distribution of cadang-cadang have been based on recognising the disease by symptoms (Price and Bigornia 1969, 1971 and 1972; Zelazny 1979; and Zelazny and Pacumbaba 1982), and the boundaries of disease incidence have been mapped (Zelazny 1979). However, the precision of such surveys is limited because of the unreliability of recognising the disease, particularly when it is at the early stage, and because of lack of data on the latent period of infection in the field. The development of biochemical methods of diagnosis by detecting the coconut cadang-cadang viroid (CCCVd) in test samples has allowed monitoring of viroid movement in the field and in experimental plots.

This chapter describes the present distribution of cadang-cadang based on assays for the viroid to identify asymptomatic palms. We have confirmed the presence of CCCVd in Southern Luzon, in isolated areas in Quezon and Aurora provinces, on neighbouring smaller islands in the provinces, on Maripipi Is., Biliran, and in Northern and Eastern Samar. The northernmost boundary of CCCVd is reported to be at General Nakar, Quezon, and the southernmost at Calicon Is., Guiuan, Eastern Samar. Long-term trials are in progress to determine the pattern of spread of the viroid.

Methods

Surveys for new cadang-cadang areas

Areas outside the previously reported northern and southern boundaries of cadang-cadang occurrence were surveyed from 1986 to 1991. Between 50% and 60% of the towns in each province were selected at random. Palms along the way to the selected sites were observed from a slow-moving vehicle or motorised boat, and whenever symptomatic palms were seen, additional sampling of this site was done. At such sites, sampling was confined to 5000 m^2

areas. A total of 1170 leaf samples were collected from 17 provinces and 5 small islands from palms that showed typical symptoms of cadang-cadang infection, from suspected diseased trees and from healthy palms. Leaflets were harvested from the second fronds of young trees and third fronds of older trees. About 10 g of sample (lamina stripped from the midrib) was sealed in polyethylene bags and stored at 4°C in the field. A maximum of 7 days was allowed between harvest of leaves and arrival at the Albay Research Centre (ARC) laboratory. The leaf samples were then stored at –20°C, extracted, and assayed by both polyacrylamide gel electrophoresis (PAGE) and molecular hybridisation assay (MHA). In some areas of Luzon and Samar, a mobile diagnostic laboratory was used for the testing of leaf samples in the field using PAGE (see Chapter 4).

Monitoring of natural disease boundaries

Since 1988, the movement of the disease has been monitored annually in three experimental plots. Two are located in areas with stable boundaries in Atimonan, Quezon, and Santa Margarita, Western Samar, and the third is in an area with actively spreading disease in Infanta, Quezon. The site in Santa Margarita is 300 m SE of the last infected palms observed in 1977. In Atimonan, the plots are 3 km NW of a small, isolated disease pocket in barangay Quilait. In Infanta, they are 6 km SW of a high incidence area.

Six 500 m × 10 m transects were established per plot so that they were perpendicular to the disease front and extended 500 m into the zone ahead of the front. The baseline reference point was the last diseased palm (i.e. the palm furthest ahead at the apparent disease front). Data from each plot were obtained only once a year because the natural spread of the disease is relatively slow. Leaf samples were collected from palms in the transects, extracted, and assayed by PAGE and MHA. Weed surveys were also conducted between the transects of the three plots.

Experimental monitoring of disease spread

Epidemiological plots were set up in November 1987 inside the ARC. Mechanically inoculated infected palms were distributed in different arrays to determine the rate, range and pattern of viroid spread into the healthy population. Zero- to 7-day-old Tambolilid coconut seedlings were mechanically inoculated with the fast electrophoretic form of CCCVd according to Imperial et al. (1985). Before field planting, all inoculated and healthy coconut seedlings were extracted and assayed by PAGE. Only those that contained the viroid were planted as infectors. These were re-assayed annually and dead infectors were replaced. Plot 1 (Fig. 5.3a) had a high density planting of infected palms throughout (25% overall incidence), and was surrounded by a row of healthy border palms. Plot 2 (Fig. 5.3b) had a central (4% overall incidence) source of inoculum, and plot 3 (Fig. 5.3c) had a peripheral (19% overall incidence) source. Each of the 1158 uninoculated palms are being extracted annually and assayed by PAGE to trace the spread of viroid from specific infectors to healthy plants.

Nucleic acid extraction and viroid detection

Leaf samples were obtained from the second- or third-youngest open frond of experimental palms. Nucleic acid extraction, preparation of dot blots on Zeta probe membrane, and viroid detection by PAGE were done following the standard procedures (Rodriguez et al. 1989; Randles et al. 1992).

The MHA was a dot blot hybridisation assay using ^{32}P-labelled RNA probe complementary to CCCVd, synthesised from an SP6-DNA clone containing a full-length insert of CCCVd, and was done by Dr D. Hanold (Hanold and Randles 1991b).

Results and Discussion

Survey of new cadang-cadang areas

The present distribution of cadang-cadang in the Philippines (Fig. 5.1) shows the northernmost boundary at General Nakar, Quezon (Lat. 14° 58'), and the southernmost at Calicon Is., Guiuan, Eastern Samar (Lat. 10° 55'). The presence of the disease in new areas was confirmed by PAGE and MHA (Table 5.1).

Table 5.1. New cadang-cadang areas in the Philippines confirmed by PAGE and MHA.

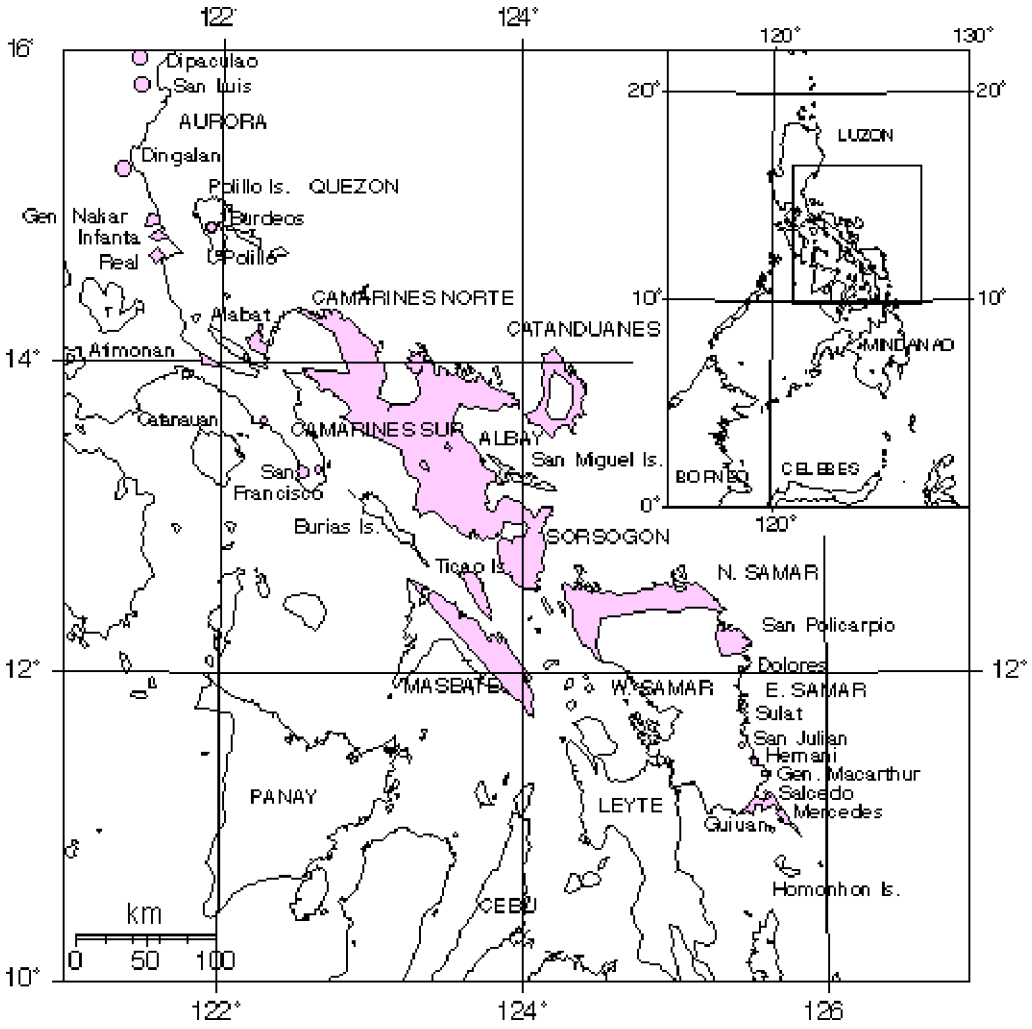
Locations	Year confirmed	Assay method	Palms containing CCCVd[1] (infected/total)		
			With CCCVd symptoms	With other abnormalities	Without symptoms
LUZON					
Quezon					
Catanauan	1989	PAGE	5/5	0/1	0/6
Polillo	1987	PAGE	1/5	–	–
Real—Balute	1987	PAGE	12/12	–	–
Real—town proper	1991	PAGE	3/3	0/1	0/11
Alabat Island	1989	PAGE	8/10	–	–
San Francisco	1989	PAGE	2/10	–	–
VISAYAS					
Eastern Samar					
Butig Is.	1987	PAGE & MHA	3/5	0/5	–
Calicon Is.	1987	PAGE	8/8	2/2	–
Dolores	1987	PAGE	8/10	–	–
Quinapondan	1987	MHA	1/3	0/2	0/5
Hernani	1987	PAGE	3/5	5	–
San Julian	1987	PAGE & MHA	2/2	1/3	0/5
San Policarpio	1987	PAGE	10/10	–	–
Sulat	1987	PAGE	2/6	0/4	–
Biliran					
Maripipi Is.	1986	PAGE	8/10	–	–

The pattern of movement of the viroid within and outwards from cadang-cadang areas varied between sites. Some sites showed expansion of the size and number of disease pockets. For example, in Capalonga, Camarines Norte, in 1979 only 1 palm in the town proper showed symptoms of cadang-cadang (Zelazny 1979), whereas in the 1989 survey 2 foci of infected palms were identified by biochemical methods: 7 palms at a site 5.5 km before the town and 49 palms in Capalonga town proper. Another example is the western part of Bondoc Peninsula,

Quezon, where the 1989 survey revealed an isolated group of infected palms 400 m NW of 3 diseased palms observed in 1982 (B. Zelazny, unpublished result from Tayuman, San Francisco). In contrast, in other areas little change in the disease boundaries had occurred (e.g. Atimonan, Quezon; Maripipi Is., Biliran; and Santa Margarita, Western Samar).

Confirmatory surveys in 1992 were conducted in the provinces of Mindoro, Central and Western Visayas, South-western Mindanao including Basilan Is., and South-eastern Mindanao. Results are yet to be confirmed by MHA.

Figure 5.1. **Distribution of cadang-cadang disease in the Philippines in 1991. (Compare with Figure 1.1, Chapter 1, which shows the year of first recording for different areas.)**



Monitoring of natural disease boundaries

The schematic layouts of the 3 experimental plots located at boundaries of cadang-cadang distribution are shown in Fig. 5.2. At the start of the trial in 1987, there were 592 experimental palms in the Atimonan plot (Fig. 5.2a). Since 1989, there has been indiscriminate cutting of older palms for coconut lumber in this plot. Four of the 181 cut palms contained the fast form of CCCVd. The reduction in the total number of experimental palms in Atimonan is shown in

Table 5.2. In the Infanta and Santa Margarita plots, no trees were cut. Most of the infected palms were in transect B in Atimonan, in transect D in Infanta, and in transect A in Santa Margarita.

Table 5.2. Number of palms containing CCCVd and percentage incidence each year in experimental plots located at boundaries of cadang-cadang occurrence. Assay by dot-blot MHA.

Location	1988		1989		1990	
	Palms with CCCVd[1] (infected/total) % incidence		Palms with CCCVd[1] (infected/total) % incidence		Palms with CCCVd[1] (infected/total) % incidence	
Quezon						
Infanta	8/467	1.7	8/467	1.7	177/467	37.9
Atimonan	6/592	1.0	4/521	0.7	25/411	6.1
Western Samar						
Santa Margarita	1/636	0.02	4/636	0.1	193/636	30.3

During 1988 and 1989, the incidence of disease did not increase in the 3 plots (Table 5.2). However, in 1990 there was a marked increase in cadang-cadang incidence in the Infanta and Santa Margarita plots and a smaller increase in Atimonan. The boundary in Atimonan seems to have been stable since 1962 (Zelazny et al. 1982).

A *Livistona rotundifolia* palm found in transect E of the Infanta plot was confirmed to be naturally infected by the viroid. This particular palm has contained the fast and slow forms of the viroid since 1987. This is the first time that a member of this species has been found to be naturally infected with CCCVd.

Of the 10 weed species found growing under coconuts located on cadang-cadang boundaries, four were predominant, based on visual observation, from 1989 to 1991. These were *Urena lobata*, *Maranta* sp., *Zingiber* sp. and *Alpinia* sp. It has been shown that some plants of these species can contain CCCVd-like molecules (M. J. B. Rodriguez, personal communication).

Experimental monitoring of disease spread

The schematic layouts of the 3 epidemiological plots using artificially inoculated infectors are presented in Figure 5.3.

Three years after field planting, all the uninoculated palms gave negative results by PAGE, indicating that there had been no spread of the viroid from infected to healthy plants. This result agrees with previous observations that, under natural conditions, palms less than 10 years old are very rarely affected by cadang-cadang. (Incidence then increases linearly up to the age of about 40 years, but then apparently remains constant in older palms (Sill et al. 1964; Zelazny and Pacumbaba 1982a).)

Four years after inoculation, infectors showed a wide range of symptom severity: about 40% showed typical disease symptoms, 7% showed the more severe brooming symptoms, 20% were dying, and 23% had only mild symptoms.

Figure 5.2. **Schematic layouts of experimental plots located at natural boundaries of cadang-cadang distribution as confirmed by PAGE and MHA. The last diseased tree (X) in the boundary is shown on transect A. Distances between transects are about 100 m.**

Figure 5.2a. **Atimonan, Quezon, with 592 experimental palms at the start of the trial in 1987. The subsequent distribution of infected palms was 6 (1988) and 4 (1989) in A; 14 (1990) in B; 6 (1990) in C; 3 (1990) in E.**

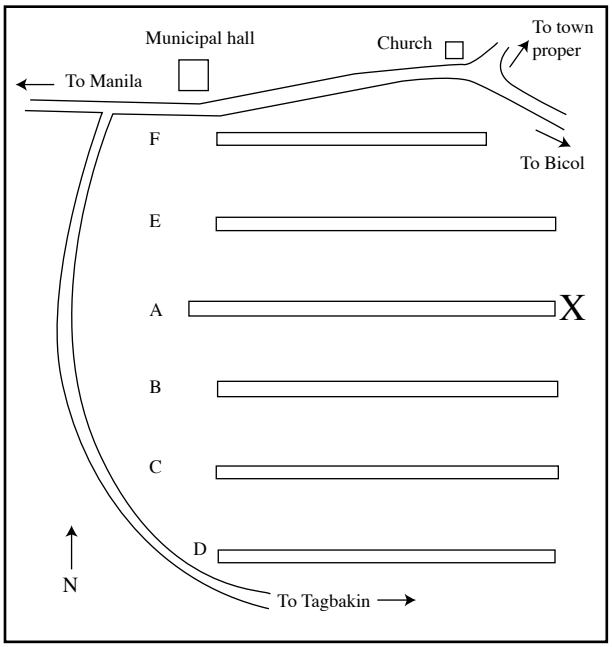


Figure 5.2b. **Infanta, Quezon, with 467 experimental palms. The subsequent distribution of infected palms was 7 (1990) in A; 5 (1990) in B; 1 (1988) and 29 (1990) in C; 3 (1988), 3 (1989) and 55 (1990) in D; 4 (1988), 4 (1989) and 34 (1990) in E; 47 (1990) in F.**

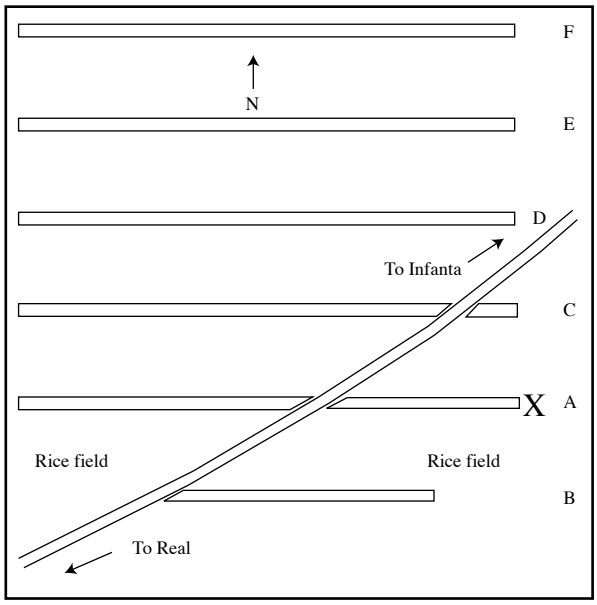


Figure 5.2c. **Santa Margarita, Western Samar, with 636 experimental palms. The subsequent distribution of infected palms was 1 (1989) and 63 (1990) in A; 1 (1988), 1 (1989) and 16 (1990) in B; 2 (1989) and 28 (1990) in C; 40 (1990) in D; 37 (1990) in E; 9 (1990) in F.**

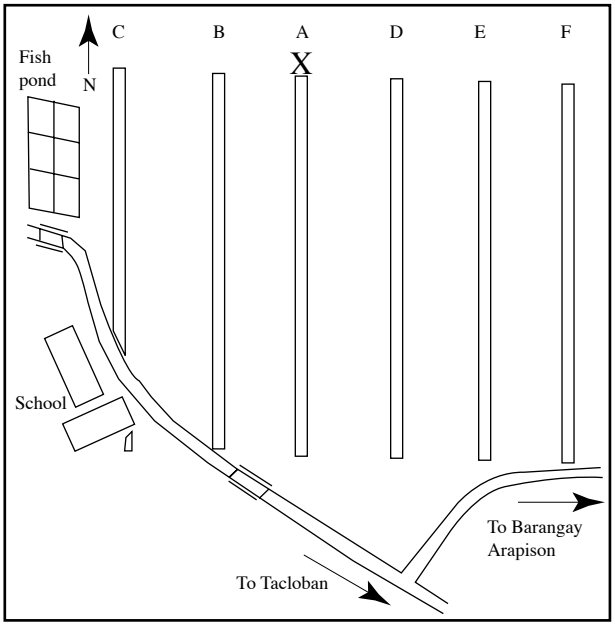


Figure 5.3. **Schematic layouts of epidemiological plots at the Albay Research Centre using artificially inoculated infectors. Crosses represent infector palms and dots indicate healthy palms. A partial layout only is illustrated.**

Figure 5.3a. **High-density planting of infectors. This plot is surrounded by 104 healthy border palms. 150 infector palms, 450 healthy palms, 25% CCCVd incidence.**

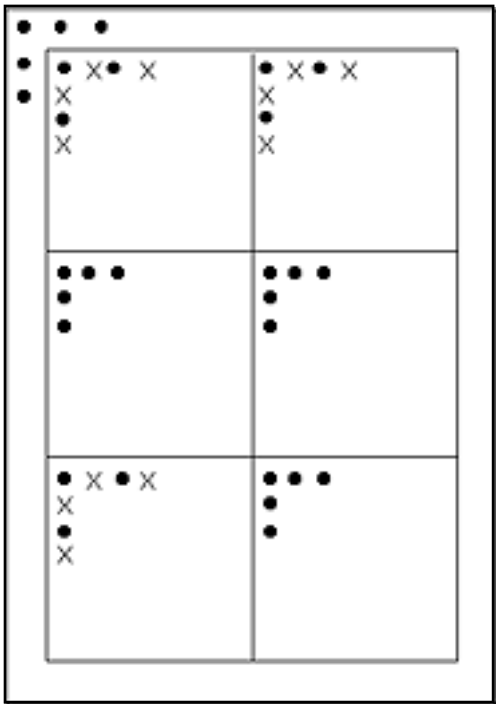


Figure 5.3b. **Central source of infection. 16 infector palms, 384 healthy palms, 4% CCCVd incidence.**

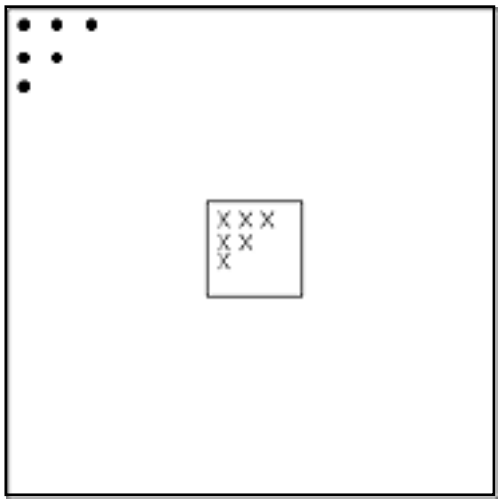
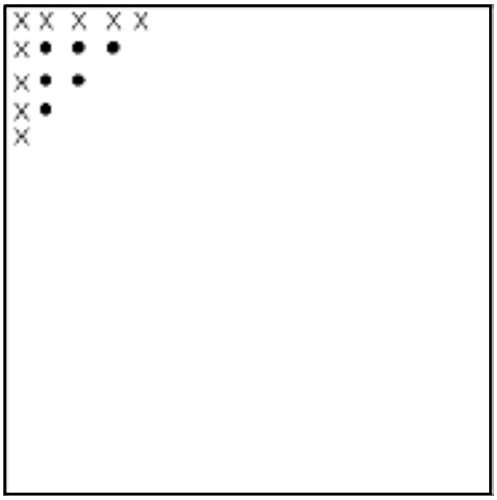


Figure 5.3c. **Peripheral source of infection. 76 infector palms, 324 healthy palms, 19% CCCVd incidence.**



At about 7 years, 25% of the 1262 uninoculated palms in the plots were producing both inflorescences and nuts, whereas only 0.8% of 242 infector palms were flowering and none produced nuts. The fertile infectors produced smaller inflorescences with time, which developed black tips and often failed to emerge fully from the covering sheath.

Acknowledgments

Support for the research was provided by the Philippine Coconut Authority and the Australian Centre for International Agricultural Research. The assistance of the technical staff and personnel of the Entomology-Epidemiology and Virology-Biochemistry Divisions is gratefully acknowledged. We wish to thank Dr Dagmar Hanold for advice during the conduct of experiments, Bong Zipagan for preparation of the map, and Eva L. Labrador for processing the manuscript.

6 LOCALISATION OF CCCVd IN POLLEN AND OTHER TISSUES OF COCONUT PALM

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Introduction

A study was conducted to determine the distribution of CCCVd in coconut palm. One of the practical aims of this work was to allow an assessment to be made of the risk of transferring CCCVd when moving germplasm or other parts of plants from sites of disease occurrence to cadang-cadang-free areas. The results of this study have implications for coconut breeders (transfer of seednuts or pollen), manufacturers of goods using coconut materials (timber, fibre, shell etc.), and producers of food items containing coconut-based ingredients.

Comparing the relative viroid content of tissues of known diseased trees may also identify parts of the plant most suitable for reliable and early biochemical detection of viroid. The fronds from the upper crown have routinely been used for assay, but they are difficult to harvest, and an aim of this work was to determine whether an alternative, reliable and easily extractable part of the tree could be identified.

Localisation in Pollen

To complement the epidemiological field studies set up to test for pollen transmission, experiments were carried out to determine whether and at what frequency the viroid could be detected in the pollen grains. Pollen of CCCVd-infected trees had previously been reported to be positive for the viroid, but as these samples contained contaminating parental tissue (such as the anthers), these experiments needed to be repeated with highly purified pollen.

Methods

The following protocol was used for preparing clean pollen from male florets:

1. Manually remove the anthers from immature male flowers (1–14 days before opening) and place in 0.1% SDS.
2. Squeeze the anthers gently in several changes of 0.1% SDS to remove the pollen grains with as little disruption to the anther tissue as possible.
3. Strain through 3 layers of fine cotton mesh.
4. Collect the pollen by centrifugation at 7000 rpm (Sorvall HS–4 rotor) for 10 minutes.
5. Remove impurities smaller than the pollen grains by layering the pollen suspension on top of 22% sucrose and allow to settle for 15–20 min. Discard the supernatant and repeat the process until all small impurities are removed (3–5 times) when checked microscopically.
6. Remove impurities larger than the pollen grains by layering the partly purified pollen suspension onto 50% sucrose and centrifuge at 1000 rpm for 15 min (Sorvall HS–4 rotor).

Two portions of pollen are obtained: the top layer, consisting of dark, mature pollen grains, and the younger grains of lighter colour suspended in the sucrose. Discard the sediment, which contains impurities such as anther tissue.

To ensure that no viroid from disrupted parental tissue could stick to the outside surface of the grains, they were treated with either 0.5 N NaOH for 1 h at room temperature, or with 8 mg/mL RNAse A for 1.5 h at 37°C. Both treatments should completely degrade any CCCVd on the outside of the grains. After the NaOH treatment, during which the colour of the pollen changed from yellow to brown, the pollen was washed with distilled water until neutral pH was reached. The RNAse treatment was followed by incubation with 2 mg/mL of protease for 1.5 h at 37°C to inactivate and degrade the RNAse.

Subsequently, the pollen was ground in a glass homogeniser in the presence of carborundum in TNEM buffer (100 mM Tris·HCl, pH 7.2, 100 mM Na acetate, 10 mM EDTA, 0.5% v/v thioglycerol) for 5 min; this broke up about 80%–90% of the grains as determined by low-power light microscopy. The homogenate was immediately extracted with 1 volume of phenol, 1 volume of chloroform and 1/10 of a volume of 10% SDS and centrifuged, and the aqueous supernatant was precipitated with ethanol. After resuspending the extract in sterile water at a ratio of 1 μL per 0.3–0.5 g pollen fresh weight, it was applied to nitrocellulose filters as a 1–2 μL dot and subjected to the hybridisation procedure using the CCCVd probe.

Results

This procedure resulted in pollen with less than 5% visible impurities as estimated by light microscopy (100 × magnification) of the 2 fractions. When checked under the microscope, neither treatment seemed to affect the structure of the pollen grains. Pollen from mixed anthers was collected for each of 22 early- to medium-late-stage cadang-cadang infected trees on the ARC station, and aliquots equivalent to 1 g were tested by dot blot hybridisation assay (see Chapter 2). Of these samples, pollen from 8 of the 22 trees were positive for CCCVd.

Comparison of Other Tissue for Analysis

Routinely, leaf blades of upper-crown fronds are used for CCCVd assay. This tissue gives reliable results and good sensitivity. However, it is difficult to obtain, especially from tall trees. A preliminary experiment was carried out with the specific purpose of identifying tissue equally suitable, but easier and faster to sample in large-scale collections, and obtainable without unduly damaging the trees.

Methods

Three cadang-cadang-infected trees (2 early, 1 medium-late stage) were selected. Tissue was taken from both the leaf blade and the central petiole of fronds 3, 7 and 12. Two trunk samples were taken per tree with a borer (18 mm diameter, 7 cm deep holes), one at about 50 cm above ground, the other 50 cm below the lowest frond. Roots have previously been shown to give unreliable results owing to heavy background staining of extracts in PAGE analysis and were therefore not included. Tissue from nut or flower areas would be just as difficult to collect as leaves and thus was not included in the assessment. Samples were extracted with the bag method (5 × 1 g lots for each sample; see Chapter 3) and analysed by PAGE assay. Three independent PAGE assays were carried out for each sample.

Results
Both the petiole and trunk extracts gave much less background in silver-stained gels than the leaf extracts. The viroid signal in the petioles was about half that in the leaves, but this disadvantage was balanced by the ease of detection of the bands due to the lower background staining. No differences in viroid amounts between samples of different fronds could be observed. The trunk assays were unreliable, as only one weak positive was found in the lower-trunk sample of the medium-stage tree.

Conclusions

In these experiments, about 30% of the pollen samples contained detectable CCCVd. Therefore, movement of pollen to cadang-cadang-free areas anywhere for breeding purposes presents a high risk of transferring CCCVd. The risk of infecting trees pollinated with this pollen has yet to be determined (but see Chapter 7).

For CCCVd assays, all the fronds gave good results, but fronds of mid-crown (around no. 12) are easier to harvest and may therefore be preferred for sampling. Petiole tissue seems just as suitable as leaf blade, and it may be easier to store and transport. Trunk samples (at least to 7 cm depth) seem to be unsuitable for assay.

So far, no part of an infected tree can be considered CCCVd-free. Moreover, CCCVd has been detected in dry husks and coconut fibre (M. J. B. Rodriguez, pers. comm.). Therefore, transfer of any kind of coconut material from infected trees must be considered a possible means of introducing CCCVd into new areas.

7 THE ROLE OF POLLEN AND SEED IN TRANSMISSION OF CCCVd

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Introduction

Some viroids can be naturally transmitted through the seed and pollen of plants (Wallace and Drake 1962; Fernow et al. 1970; Singh 1970; Singh and Finnie 1973; Desjardins 1987; Kryczynski et al. 1988). Coconut cadang cadang viroid has been found to be seed-transmissible at a rate of about one in 300 (B. Zelazny and E. Pacumbaba, cited in Randles and Imperial 1984). It has been detected in the husk (M. J. B. Rodriguez, unpublished) and embryo (D. Hanold, unpublished) of coconuts, and has also been detected in the purified pollen of diseased palms (see Chapter 6).

In line with these previous observations, this study was set up to determine whether the pollen or seed is involved in the natural spread of the cadang-cadang disease.

This chapter describes a trial in which the transmission of coconut cadang-cadang viroid (CCCVd) through pollen and seed was investigated. A controlled hand-pollination technique was used to regularly pollinate 120 emasculated healthy mother palms. The mother palms were checked for infection via the embryo or endosperm, and the F1 progeny were checked for seedborne infection. We report that 2 mother palms became viroid-positive 1.5–2 years after start of pollination, one by healthy dried pollen artificially contaminated with purified CCCVd, the other by 'infected' dried pollen also artificially contaminated with purified CCCVd.

Methods

Identification of mother palms and pollen source

Bearing Laguna Tall palms inside the Albay Research Center were screened for the presence of CCCVd at frond no. 3 by PAGE assay (PCA 1986). One hundred and twenty cadang-cadang-free mother palms were selected. Some of the healthy palms used in treatments HDP and HFP (see below) were used as sources of healthy pollen. Palms at the early stage of the disease containing the 246/247 nucleotide forms of CCCVd were used as sources of infected pollen. These palms were outside the Center. When donor palms stopped pollen production, they were replaced by other early-stage trees. Overall, a total of 34 diseased palms were used as a pollen source.

Inoculum preparation

The method of Imperial et al. (1985) was used to prepare CCCVd inoculum. About 100 kg of leaves from identified diseased palms containing the 246/247 nucleotide forms of the viroid were extracted.

Pollen collection and pollination

All methods were based on those described by Balingasa and Santos (1978). Pollination of each inflorescence was started during the receptive phase of the female flowers (buttons) and on 2 consecutive days thereafter.

Experimental treatments

The following treatments and pollen sources were used:

- healthy dried pollen (HDP)
- infected dried pollen (IDP)
- healthy dried pollen + purified CCCVd (HDP + Vd)
- infected dried pollen + purified CCCVd (IDP + Vd)
- healthy fresh pollen (HFP)
- infected fresh pollen (IFP).

Twenty mother palms were pollinated per treatment. About 25 mg of partially purified CCCVd was added per inforescence to pollen for treatments HDP + Vd and IDP + Vd.

Assay by PAGE and MHA

To assess whether transmission had occurred, the mother palms were assayed by PAGE for the presence of CCCVd once a year.

Ten to 12 months after pollination, F1 seednuts were harvested and partially dehusked before sowing in the nursery. Five-gram samples from the removed husk material were extracted using the Na_2SO_3–PEG method and assayed. Germination of seednuts was monitored daily. The seedlings were assayed by testing the viroid content of frond no. 2 six months after germination. After planting in the field, seedlings were assayed every 6 months.

Results and Discussion

Pollination, nut collection and nut germination

From 1987 until the third quarter of 1992, 5128 inflorescences were pollinated and 3476 mature F1 seednuts were harvested (Table 7.1). Higher percentages of germination were observed for nuts produced by pollination with fresh pollen than by dried pollen; this suggests a decrease in pollen viability during the drying process. No significant trend in germination rate of F1 progenies was noted when diseased pollen was used compared with healthy pollen (Table 7.1).

Table 7.1. **Number of inflorescences pollinated, nuts harvested and nuts germinated (from 1987 to 3rd quarter 1992).**

Treatment	Number of pollinated inflorescences	No. of nuts harvested	% germination	per inflorescence	germinated
HDP	953	529	0.6	281	53
IDP	806	379	0.5	207	55
HDP + Vd	908	485	0.5	256	53
IDP + Vd	742	296	0.4	148	50
HFP	924	1004	1.1	797	79
IFP	795	783	1.0	679	87
Total	5128	3476		2368	

Detection of CCCVd in mother palms

The Bicol region of the Philippines was badly hit by typhoons Herming (August 1987) and Sisang (November 1987), which killed 42 of the mother palms and caused severe abortion of nuts. An attempt was made to raise 223 embryos from the prematurely fallen nuts in vitro, but none survived.

Assays of the remaining 78 mother palms showed that one each from treatments HDP + Vd and IDP + Vd were positive for CCCVd (Table 7.2). The palms were positive for CCCVd by dot-blot MHA in 1988 and electroblot MHA in 1989 (ARC 1990). Moreover, these palms were positive for CCCVd when all mother palms were assayed by PAGE in 1990 (ARC 1990) and in June 1992.

Table 7.2. **Results of nucleic acid assay of 78 surviving mother palms in the pollen transmission experiment.**

Treatment	Date of first pollination of mother palm positive for CCCVd	Infected /total	Assay done	CCCVd form in PAGE
HDP		0/15	PAGE (1990)	none
			PAGE (1992)	none
IDP		0/11	PAGE (1990)	none
			PAGE (1992)	none
HDP + Vd	2 February 1987	1/15	dot-blot MHA (1988)	–
			PAGE (1990)	246
			PAGE (1992)	246, 247, 296
IDP + Vd	25 February 1987	1/10	electroblot MHA (1989)	–
			PAGE (1990)	246 f int.
			PAGE (1992)	297, s int.
HFP	9 February 1987	*1/5	PAGE (1990)	none
			PAGE (1993)	246
IFP		0/12	PAGE (1990)	none
			PAGE (1992)	none

*Symptomless; possible natural infection not associated with experimental pollination.

Detection of CCCVd in F1 progeny

Assays of husks for CCCVd showed that a low percentage of nuts from all treatments were positive for CCCVd. Higher percentages of infection were observed in husks from the treatments using diseased pollen (Table 7.3). The percentage infection using infected dried pollen + purified CCCVd was more than double that for healthy dried pollen + purified CCCVd. The number of husks positive for CCCVd was also higher in treatments using infected rather than healthy pollen, either fresh or dried. Assay results of leaf samples from frond no. 2 of germinated F1 seedlings showed the highest percentage of infection from infected fresh pollen (Table 7.3). However, the presence of CCCVd in husks and leaf samples in treatments using either fresh or dried healthy pollen could not be explained.

Table 7.3. Results of assay of nucleic acid extracts from husks of the harvested nuts and leaf samples of frond no. 2 of germinated F1 progenies (from 1987 to 3rd quarter 1992).

Treatment	Infection			
	Husks		Frond no. 2	
	Infected/total	%	Infected/total	%
HDP	30/529	5.7	8/281	2.9
IDP	33/379	8.7	0/207	0
HDP + Vd	28/485	5.8	6/256	2.3
IDP + Vd	37/296	12.5	3/148	2.0
HFP	23/1004	2.3	9/797	1.1
IFP	31/783	4.0	23/679	3.4

Of the total 2368 nuts that had germinated, 631 have so far been field-planted in blocks (25 test palms per treatment per block). Six of these palms were positive for CCCVd when assayed by PAGE in 1992 (1 palm from treatment IDP + Vd and 5 palms from treatment IFP). The 5 palms from treatment IFP were all the progeny of one single mother palm negative for CCCVd by PAGE assay. The mother palm of the progeny positive for CCCVd from treatment IDP + Vd was also negative for CCCVd. The interpretation of these results is not clear, but the implication that viroid may be passed to seedlings without the parent showing infection needs to be further investigated. The trial is continuing.

Acknowledgments

The authors acknowledge the support of the Philippine Coconut Authority and the Australian Centre for International Agricultural Research (ACIAR) in the conduct of this study. Special thanks to Cristeta A Cueto, Maria Teresa I. Namia, Pilar L. Tocino and the staff of the Virology-Biochemistry Division.

8 STUDIES OF DISEASE TRANSMISSION BY INSECTS

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Introduction

The destructive nature of coconut cadang-cadang disease has been well documented. Its slow movement out of formerly identified areas (ARC 1987, 1988, 1989, 1990) remains a threat to coconut-growing and oil-palm-growing regions of the world. The mode of spread remains unknown despite numerous and extensive studies.

From the time of discovery of the disease until the late 1970s, numerous insect transmission trials have been done covering more than a hundred species of Heteroptera, Coleoptera and Orthoptera. The trials were done solely with field-collected insects from high-incidence areas. No inoculated test plants became infected with cadang-cadang in these trials, with the result that no insect vector could be implicated in the spread of the disease (FAO 1982).

In recent years, the nature of pathogen–insect vector relationships has become better understood, and this information is used in devising the best methods of crop protection. Numerous insects from 7 taxonomic orders, as well as mites, nematodes and fungi, have been implicated in the spread of various plant viral diseases (Fulton et al. 1987; Thresh 1974), and aphids may be responsible for the field spread of at least one viroid in tomato (De Bokx and Pirou 1981).

The present work deals with a study of the potential of several species of insects closely associated with coconut palms to transmit CCCVd.

Methods

Insect survey

In 1981, a widespread insect survey program was started to determine which species were most commonly associated with high incidence of the disease. Thirty-six sites with differing disease intensity (zero, low, medium and high) were selected from provinces with cadang cadang in the Bicol, Quezon and Samar regions of the Philippines. Eight each of healthy young (< 10 years old) and tall (– 20 years old) palms were selected per site and surveyed 3 times at different times of the year. Sampling methods were sticky trapping, live collection and net sweeping under coconut plantations. Preliminary identification of insect collections was done to the level of family.

Mass rearing

Mass-rearing methods of suspected insect vectors were studied to provide disease-free populations for transmission work. Adult beetles were collected from healthy palms and reared under artificial conditions.

Plesispa reichei Chapuis

Field-collected adults fed and laid their eggs in coconut leaflets. Fifty adults were introduced to 60 leaflets that were bundled and enclosed in a cage made of fine mesh cloth. Leaflets were positioned upright and their ends were immersed in water. After 20 days, egg-laying adults were transferred to fresh leaves and larvae were placed in petri dishes lined with moist tissue paper. Larvae were maintained in leaflets cut into 5 cm pieces arranged in layers. Dry leaves were replaced twice a week until the larvae pupated. Pupae were pooled in petri dishes lined with tissue paper until the adult stage emerged.

Octodonta angulosa Uhmann

Leaves of buri palm (*Corypha elata*) were pre-soaked overnight in water and cut into 8 cm pieces and placed in rearing bottles lined at the bottom with moist tissue paper. Mixed populations of 50 field-collected adults were introduced to each bottle for egg laying. After 10 days, dry leaves were replaced and larvae were collected. The larvae were maintained under the same conditions until pupation. Pupae were maintained inside the folds of buri leaves until the adult stage emerged.

Test of viroid-vector interaction

Vector-mediated experiments were conducted using 2 beetle species.
P. reichei Chapuis

In the control treatment, beetles were given acquisition access feeds (AAF) on healthy coconut palms. In the other 3 treatments, beetles had 2, 12 or 48 h of acquisition access on random fronds of mechanically inoculated, field-planted coconut palms containing the 246/247 form of CCCVd. Following the access period, inoculative insects were transferred to healthy coconut seedlings (3-leaf stage) maintained in insect-proof cages at about 20–25 insects per plant and allowed to feed for 5 days. In each experiment, 10 host plants for each of the treatments were inoculated 3 times and the treatments were replicated 6 times. Each treatment used 1200 insects. Host plants were assayed 1 year after the last inoculation.

O. angulosa Uhmann

The insects in this trial had the same acquisition access periods as above. The 2- and 48-h acquisition feeds were set up on frond no. 2 of mechanically inoculated coconut palms containing the 246/247 nucleotide form of CCCVd, and the 12-h access feeds were set up on frond no. 1 of the same palms.

Test for uptake of CCCVd by insects

Two beetle species, *Derelomorphus* sp. and *Promecotheca* sp., were collected from healthy and diseased coconut palms. *P. reichei* and *O. angulosa* were also collected, but since these two were successfully reared, infectivity tests included both oral inoculation of insects with purified CCCVd extracts and a 24 h AAF on mechanically inoculated coconut palms containing the 246/247 nucleotide form of CCCVd. The insects were extracted and assayed by molecular hybridisation. Whole insects were weighed (*Derelomorphus* sp. = 0.01 g; *Promecotheca* sp.

= 0.40 g; *Plesispa* = 0.30 g; and *Octodonta* = 0.30 g) and placed in Eppendorf tubes containing 1.5 mL of AMES buffer (see Chapter 3); a further 4 mL of AMES was added per gram of insect, and the samples were ground using a glass rod. Aqueous 90% phenol was added at 2 mL/g, and samples were incubated for 15 min in a 37°C water bath and then shaken for 15 min. An amount of chloroform equal to the phenol was added, and the mixture was shaken vigorously for 15 min and centrifuged. The nucleic acids in the supernatant were ethanol-precipitated, resuspended in sodium acetate – sucrose, dot-blotted onto Zeta probe nylon membrane (Biorad), and hybridised with a ^{32}P-labelled radioactive cRNA probe by D. Hanold (Hanold and Randles 1991b).

Viroid detection in host plants

One-gram samples of coconut leaves from frond 2 of host palms were individually crushed and extracted following the method described by Rodriguez et al. (1989). The resulting nucleic acids were analysed by gel electrophoresis in 20% polyacrylamide gels (Imperial et al. 1985).

Results and Discussion

Insect survey

In the recently concluded insect survey of coconut plantations, 5 species from the Coleoptera and 9 species from the Heteroptera were frequently collected from the 36 sites (Table 8.1). Nine sites were identified for each of the following levels of disease incidence: zero, low (< 2%), medium (< 15%) and high (> 15%). Analysis of insect numbers according to disease level is still in progress.

Table 8.1. Insect populations occurring on diseased and healthy coconut palms surveyed in South-eastern Luzon and North-western Samar, ranked according to abundance. (Total numbers at 36 sites of 100 palms each with varying cadang-cadang incidence.) Analysis of possible correlation between insect numbers and disease level is still in progress.

Insect species	Insect order	No. of individuals on young palms (< 10 y old)	No. of individuals on tall palms (> 20 y old)	Total
Bakera nigroescuta	Heteroptera	2478	1068	3546
Cerataphis lataniae	Heteroptera	1033	1352	2385
Aspidiotus destructor	Heteroptera	1029	878	1907
Derelomorphus sp.	Coleoptera	97	1474	1571
Plesispa reichei	Coleoptera	317	276	593
Aleurodicus destructor	Heteroptera	508	23	531
Hemipeplus sp.	Coleoptera	80	343	423
Promecotheca similis	Coleoptera	230	139	369
Bothrogonia longa	Heteroptera	318	35	353
Stephanitis typica	Heteroptera	220	41	261
Virgilia luzonensis	Heteroptera	130	27	157
Oryctes rhinoceros	Coleoptera	45	109	154
Promecotheca cumingi	Coleoptera	54	70	124
Virgilia sp.	Heteroptera	19	15	34

Insect counts were recorded separately for mature palms and for pre-bearing palms (< 10 years old), which do not normally show the disease. A higher number of individuals on mature palms could point towards a possible involvement of this particular species in natural CCCVd transmission.

The insects were observed feeding on coconut (some actually bred) and were apparently abundant. Statistical analyses of survey data are being done to correlate insect populations with disease incidence, elevation and palm age, and thus to identify species that could possibly transmit the disease and that are suitable for use in inoculation trials.

Mass rearing

From the insects collected and identified, 2 species of beetles were successfully mass-reared under our laboratory conditions: *O. angulosa* and *P. reicheii*.

O. angulosa was occasionally collected from coconut palms but is conspicuously associated with buri palms, a natural host of the viroid. This species was found to contain CCCVd in a preliminary assay of insects collected from infected palms. Although this may have been due to sap in the gut and is no proof of vector activity, the species was included in mass rearing and insect transmission studies.

Studies to establish mass-rearing methods for a lacebug and 3 other beetles are continuing. Among these is *Derelomorphus* sp., a tiny apionid beetle that was found feeding on pollen and breeding on male flowers of coconut.

Viroid–vector interaction

Transmission trials using *Plesispa* as a vector have been concluded (Table 8.2) but results are preliminary. Two test plants each of treatment 1 (AAF on healthy palms) and treatment 4 (48 hr AAF on CCCVd-infected palms) were positive for the viroid 1 year after inoculation as detected by dot-blot molecular hybridisation assay. PAGE assays of the same trees were negative. Interpretation of these results is not clear, and this species is to be tested further for its ability to transmit CCCVd. Transmission experiments using Octodonta (Table 8.3) have not yet been analysed.

The first replicate of tests for CCCVd uptake by *Plesispa* and *Octodonta* included insects collected from both diseased and healthy palms, insects fed on infected and healthy palms, and insects inoculated orally with purified CCCVd extracts (4 μg of viroid/10 μL). Extracted samples were dot-blotted onto Zeta probe membrane for hybridisation (Table 8.4). Results are not yet available.

Table 8.2. Transmission experiments using *P. reichei* Chapuis as vector.

Treatment	No. replicates	Total no. inoculated host plants	Total no. insects used	Total no. positive host plants
0. Control	6	60	0	0
1. Healthy	6	60	1200	2/60*
2. 2 h AAF	6	60	1200	0
3. 12 h AAF	6	60	1200	0
4. 48 h AAF	6	60	1200	2/60*

* Preliminary results; confirmation required.

Table 8.3. Transmission experiments using *O. angulosa* Uhmann as vector.

Treatment	No. replicates	Total no. inoculated host plants	Total no. insects used	Total no. positive host plants
0. Control	7	70	0	*
1. Healthy	7	70	1400	
2. 2 h AAF	7	70	1400	
3. 12 h AAF	7	70	1400	
4. 48 h AAF	7	70	1400	

* First assay was done only on first replicate. All other test plants will be assayed 1 year after the host inoculation.

Table 8.4. Molecular hybridisation assay of insects for CCCVd-uptake.

Insect species	Weight of sample (g)*	No. samples extracted†	No. replicates	Results§
Promecotheca sp.	0.4	D = 80	8	
		H = 80	8	
Derelomorphus sp.	0.1	D = 60	6	
		H = 60	6	
Plesispa reichei	0.3	T1 = 10	1	
		T2 = 10	1	
		T3 = 10	1	
		T4 = 10	1	
		T5 = 10	1	
Octodonta angulosa	0.3	T1 = 10	1	
		T2 = 10	1	
		T3 = 10	1	
		T4 = 10	1	
		T5 = 10	1	
		T6 = 10	1	

* Consisting of varying numbers of insects, depending on size of individuals.
†D = collected from diseased palms; H = collected from healthy palms; T1–6 = treatments: see text.
§Not available.

[Editors' note: Although Tables 8.2, 8.3 and 8.4 are incomplete, they are the only record of the species used and how the experiments were done. The trees that were inoculated are still growing; results will be monitored over the next few years.]

The search for vectors is a complicated undertaking since it is affected by numerous factors. For instance, insect surveys to provide data as a basis for vector screening produce voluminous collections that have to be identified. The disease also has a long incubation period in the host plant. There is an irregular association between suspected vectors and host plants, and so far possible low frequencies of inoculation by insects that had fed on diseased plants have been observed.

9 SCREENING OF COCONUT POPULATIONS FOR RESISTANCE TO CCCVd USING COCONUT SEEDLINGS

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Introduction

Many viroids can be mechanically transmitted (Diener 1979). In the case of cadang-cadang, the viroid was first successfully transmitted to 4–18-month-old coconut seedlings by high-pressure injection combined with either rubbing with carborundum or slashing of petioles with a razor (Randles et al. 1977). A later study showed that transmission could be achieved by high-pressure injection of nucleic acid extracts into younger seedlings (Randles et al. 1980b). The rate of transmission can be increased by increasing the amount of viroid injected (Zelazny et al. 1982), either by increasing RNA concentration in the inoculum or by increasing the number of injections (PCA 1987). CCCVd could be detected by PAGE as early as 6 months after inoculation (PCA 1987).

Resistance to inoculation by high-pressure injection of viroid was evaluated in a range of coconut populations to estimate their relative susceptibility to cadang-cadang and to identify populations that may be resistant to infection with the viroid.

Methods

Source of inoculum

Naturally infected coconut palms containing the 246/247 forms of CCCVd (early stage) were used as sources of infected tissue (Mohamed et al. 1985). Leaflets were stripped from the petiole, midribs were removed and the leaf blades were chopped into small pieces with a chaff cutter.

Preparation of inoculum

Inoculum was prepared as described by Imperial et al. 1985. Nucleic acids were extracted by blending chopped leaflets in 0.1 M Na_2SO_3 (700 mL/250 g leaflets) followed by concentration with polyethylene glycol (PEG) mol wt 8000 (75 g/L). Purification of nucleic acids from the PEG pellet was done by repeated extraction with a 2:1:1 mixture of sodium dodecyl sulphate (SDS) (10 g/L), phenol (900 g/L) and chloroform (Randles 1975b; Imperial et al. 1981). For a PEG pellet equivalent to 5 kg of leaf, 80 mL 1% SDS, 40 mL phenol and 40 mL $CHCl_3$ were used. Nucleic acids were recovered from the aqueous phase by precipitation with three volumes of ethanol in the presence of 0.1–0.3 M sodium acetate. The ethanol precipitate was vacuum-dried, dissolved in 0.1 M sodium acetate, and then divided into 1 mL aliquots. The total RNA concentration was determined by absorption measurement. To obtain the required CCCVd concentration needed for inoculation, the inoculum was dried and dissolved in 1 × SSC (0.15 M NaCl; 0.5 M Na citrate) to give a concentration of 20 mg/mL.

Source of seednuts for inoculation

Except for Tambolilid (TAMB), all coconut populations for resistance screening trials were provided by the Philippine Coconut Authority's Zamboanga Research Center. Tambolilid nuts were obtained from Daet, Camarines Norte. Mother palms were assayed by PAGE to ensure that they were CCCVd-free.

Inoculation procedure

Using a hand-primed, high-pressure Panjet injector, 0–7-day-old sprouts emerging from trimmed seednuts were inoculated at the base with a total of 4 μg CCCVd per sprout for every inoculation. Two injections each of 0.1 mL were applied per inoculation, and the inoculation was repeated twice at intervals of about 17 days. After the first inoculation, seedlings in the nursery were planted in polybags until the first assay and then field-planted (PCA 1985, 1987).

Populations that included pure lines and hybrids were tested in groups of 5–7, with 10–20 sprouts per population replicated 2–4 times. Tambolilid, a commonly planted variety in the Bicol Region of the Philippines and previously identified as susceptible to cadang-cadang, was used as the reference cultivar.

Maintenance of the test seedlings

Field-planted seedlings were maintained under optimal cultural conditions, which included fertilising, ring-weeding and harrowing.

Assay and analysis of data

The test seedlings were assayed for the presence of CCCVd 6 months after inoculation and every 6 months thereafter by polyacrylamide gel electrophoresis (PAGE) (Imperial and Rodriguez 1983). Three years after inoculation, a molecular hybridisation assay (MHA) (Thomas 1980; Owens and Diener 1981; Mohamed and Imperial 1983; Symons 1984) was done. Palms found to be negative for CCCVd by MHA were reinoculated and assayed 6 months afterwards by PAGE, MHA or both.

The average percentage of infection per assay for every batch of inoculated populations was calculated.

Results and Discussion

Screening for resistance against cadang-cadang disease by mechanical inoculation of sprouts on 82 coconut populations, including Tambolilid as control, was evaluated (Table 9.1).

Preliminary experiments with mechanical inoculation of CCCVd were done on 5 coconut populations: Alsua Survivors, Catigan, Malayan Yellow Dwarf, Malayan Red Dwarf and Tambolilid. Ten sprouts per population were inoculated once only; there were 7 replicates. The inoculated seedlings were assayed yearly by PAGE (Table 9.2). Statistical analysis revealed no significant differences in the percentages of infection.

Slightly higher transmission was achieved by 3 sequential mechanical inoculations on 7 populations: 60%–90% 6 months after inoculation compared with 70%–80% 1 year after

infection by single inoculation (PCA 1987). The remaining populations (Table 9.3) were therefore screened using the 3 sequential CCCVd injections (PCA 1987).

The variability in the infection rate of the reference cultivar Tambolilid in the experiments is given in Table 9.4.

After 7 years, no resistant population could be identified. However, among the plants initially inoculated only once with CCCVd, 14 individual palms remained negative for the viroid by both PAGE and electroblot MHA (January 1991), even after 2 further 3-sequential inoculations, carried out in September 1988 and in June 1990. Whole-crown PAGE assays done in February 1992, however, detected 4 of these palms as positive for CCCVd (Table 9.5), hence reducing the number of negative palms to 10. The 10 belong to the following coconut populations: 3 Alsua Survivors, 2 Malayan Yellow Dwarf, 1 Malayan Red Dwarf and 4 Tambolilid.

Visual assessment showed that the 10 palms grew normally. The 4 palms recently found positive are under observation to determine whether they will develop disease symptoms.

Progeny of the 10 palms remaining CCCVd-negative will be used as populations in the continuing screening program. It is planned to self the palms, and to inoculate half of the harvested nuts and plant the others without treatment for future monitoring of their CCCVd content.

Considering that the cultivars to which these palms belong do not generally exhibit genetic resistance, possible reasons for the inoculated palms remaining CCCVd-free need to be investigated. They could include, for example, genetic mutations occurring in the individual plants, or the possibility of the disease entering a stage of remission, a phenomenon that has been reported for some viruses. In the first case, selfed progeny would retain CCCVd resistance and give rise to a new breeding line. In the case of remission, not much is known about the mechanisms of its occurrence and how it is triggered, but it cannot be expected to be passed on to progeny. The possibility of cadang-cadang going into remission would also explain why, in many disease areas, ‘survivors’ have occasionally been observed (B. Zelazny, pers. comm.); that is, individual trees that did not perish after all other palms had died, but did not give rise to resistant progeny when selfed. To investigate whether the stage of remission could be artificially induced and thus be used as a control measure would make a worthwhile subject for future research.

Acknowledgments

We acknowledge the support of the Philippine Coconut Authority and the Australian Centre for International Agricultural Research (ACIAR) in the conduct of this study. Special thanks to Bernarda Mercado, Maryjane B. Orolfo and the staff of the Virology-Biochemistry Division, Albay Research Center.

Table 9.1. Coconut populations screened for resistance against cadang-cadang disease 1985–1991.

Tall	Dwarf	Hybrid	
Aguinaldo (AGI)	Aromatic (ARO)	BAO × BAO	MRD × PYT
Alsua Survivors*	Baguer (BAG)	BAO × CRD	MRD × TAC
Bago-Oshiro (BAO)	Banga (BAN)	BAO × WAT	MRD × TAG
Baybay (BAY)	Catigan (CAT)	BAY × CRD	MRD × TAH
Gatusan (GAT)	Cameroon Red (CRD)	BAY × SGD	MYD × LAG
Gazelle (GPT)	Equatorial Green (EGT)	BAY × WAT	MYD × PYT
Hijo (HJT)	Galas (GAL)	CAT × BAO	MYD × SNR
Karkar (KAR)	Kapatagan (KAP)	CAT × CAT	MYD × TAG
Laguna (LAG)	Kinabalan (KIN)	CAT × LAG	MYD × WAT
Markham Valley (MVT)	La Victoria (VIC)	CAT × PYT	RIT × CRD
Polynesian (PYT)	Magtuod (MAG)	CAT × RIT	RIT × SGD
Rennell (RT)	Makilala (MAK)	CAT × TAG	RIT × TAC
San Ramon (SNR)	Malayan Yellow (MYD)	CAT × WAT	RIT × WAT
Spicata (SPI)	Malayan Red (MRD)	CRD × WAT	SNR × WAT
Tagnanan (TAG)	Pilipog (PIL)	EGD × WAT	TAC × BAO
Zamboanga (ZAM)	Sri Lankan (SGD)	LAG × CRD	TAC × BAY
	Tacunan (TAL)	LAG × LAG	TAC × CRD
	Tambolilid (TAMB)	LAG × WAT	TAC × LAG
		MAT × CRD	TAC × RIT
		MAT × MYD	TAC × TAC
		MRD × BAO	TAC × TAG
		MRD × BAY	TAC × WAT
		MRD × CAT	TAG × WAT
		MRD × LAG	ZAM × WAT

* Progeny of survivor palms on Ramon Alsua's farm, Paulog, Ligao, Albay.

Table 9.2. Percentages of infection based on yearly PAGE assay of coconut populations inoculated once with CCCVd. Average of 7 replicates with 10 palms per population per replicate.

Population	Infection per assay											
	1986		1987		1988		1989		1990		1991	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Alsua	50/70	71	63/70	90	62/70	90	65/70	93	66/70	94	66/70	94
CAT	51/70	73	65/70	93	65/70	93	69/70	99	69/70	99	69/70	99
MRD	56/70	80	63/70	90	65/70	93	67/70	96	69/70	99	69/70	99
MYD	46/70	66	63/70	90	63/70	90	65/70	93	66/70	94	66/70	94
TAMB	54/70	77	63/70	90	63/70	90	65/70	93	65/70	93	65/70	93
Mean		73		91		91		95		96		96
Statistical significance	NS		NS		NS		NS		NS		NS	
Standard error	8.38		4.11		3.83		2.27		2.02		2.02	
C.V.%	30.2		12.0		11.1		6.4		5.6		5.6	

Table 9.3. Susceptibility of coconut populations screened for resistance to CCCVd using PAGE after mechanical inoculation.

Variety	Date of inoculation	No. of assays	Infection		Tambolilid control		% variety / % Tambolilid
			Infected/ total	%	Infected/ total	%	
Alsua Survivors	7/86	7	67/70	96	66/70	94	1.02
BAY	1/87	7	30/30	100	30/30	100	1.00
CAT	1/87	7	30/30	100	30/30	100	1.00
LAG	1/87	7	29/29	100	30/30	100	1.00
MAK	1/87	7	29/30	97	30/30	100	0.97
MYD	1/87	7	30/30	100	30/30	100	1.00
MRD	1/87	7	28/28	100	30/30	100	1.00
CAT × LAG	4/88	7	29/40	73	23/40	58	1.26
MRD × BAY	4/88	7	20/40	50	23/40	58	0.87
	5/89	6	13/20	65	19/20	95	0.63
MYD × WAT	4/88	7	31/40	78	23/40	58	1.35
	1/91	3	20/20	100	19/20	95	1.05
TAC × WAT	4/88	7	31/40	78	23/40	58	1.35
	5/89	6	20/20	100	19/20	95	1.05
BAO	8/88	6	30/40	75	30/40	75	1.00
HJT	8/88	6	31/37	88	30/40	75	1.12
RIT	8/88	6	25/40	63	30/40	75	0.83
SNR	8/88	6	27/40	68	30/40	75	0.90
	6/89	5	18/20	90	7/16	94	2.06
TAG	8/88	6	31/40	78	30/40	75	1.03
	5/89	6	18/19	95	19/20	95	0.99
ZAM	5/89	6	14/20	70	19/20	95	0.74
TAC × BAO	5/89	6	11/13	85	19/20	95	0.89
CAR × PYT	5/89	6	16/20	80	19/20	95	0.84
MRD × TAG	5/89	6	19/19	100	19/20	95	1.05
	5/89	6	15/15	100	19/20	95	1.05
MRD × CAT	5/89	6	16/20	80	19/20	95	0.84
MRD × BAD	5/89	6	19/20	95	19/20	95	1.00
CAT × BAO	5/89	6	15/19	79	19/21	95	0.83
KAR	5/89	6	18/20	90	19/20	95	0.95
CAT × TAG	5/89	6	19/20	95	19/21	95	1.00
MRD × LAG	5/89	5	18/20	90	7/16	44	2.06
BAG	5/89	5	20/20	100	7/16	44	2.29
EGC	5/89	5	7/9	78	7/16	44	1.78
BAO × BAO	6/89	5	15/20	75	7/16	44	1.71
GAL	6/89	5	11/20	92	7/16	44	2.09
LAG × LAG	6/89	5	8/20	40	7/16	44	0.91
CAT × CAT	6/89	5	14/20	70	7/16	44	1.60

Table 9.3. (cont'd) Susceptibility of coconut populations screened for resistance to CCCVd using PAGE after mechanical inoculation.

TAC × TAC	6/89	5	14/20	80	7/16	44	1.83
MYD × SNR	6/89	5	17/20	85	7/16	44	1.94
TAL	6/89	5	9/6	67	7/16	44	1.52
TAC × BAY	6/89	5	17/20	85	7/16	44	1.94
TAC × LAG	6/89	5	15/20	75	7/16	44	1.71
MYD × LAG	7/89	5	15/20	75	7/16	44	1.71
KIN	7/89	5	14/30	47	7/17	41	1.13
PIL	7/89	5	15/21	71	7/17	41	1.73
TAH	7/89	5	11/28	39	7/17	41	0.95
TAC	8/89	5	18/30	60	7/17	41	1.47
BAO × WAT	1/91	3	13/91	69	15/06	94	0.73
BAY × WAT	1/91	3	13/20	65	15/16	94	0.64
EGD × WAT	1/91	3	13/21	62	15/16	94	0.66
LAG × WAT	1/91	3	17/20	85	15/16	94	0.91
MAT × CRD	1/91	3	12/20	60	15/16	94	0.64
MAT × MYD	1/91	3	8/13	62	15/16	94	0.66
RIT × TAC	1/91	3	16/20	80	15/16	74	0.85
RIT × WAT	1/91	3	19/20	70	15/16	94	0.75
SNR × WAT	1/91	3	9/19	47	15/16	94	0.50
TAG × WAT	1/91	3	9/20	45	13/16	94	0.48
ZAM × WAT	1/91	3	10/19	53	15/16	94	0.56
CRD × WAT	1/91	3	17/20	86	15/16	94	0.91
AGI	1/91	3	11/16	69	8/15	53	1.29
GAT	1/91	3	9/16	56	8/15	53	1.06
MYD × PYT	1/91	3	13/20	65	8/15	53	1.22
MYD × TAG	1/91	3	12/12	57	8/15	53	1.02
SPI	1/91	3	19/19	100	8/15	53	1.88
MAG	2/91	3	12/16	75	8/15	53	1.41
RIT × CRD	2/91	3	12/20	60	8/15	53	1.12
RIT × SGD	2/91	3	16/20	80	8/15	53	1.50
TAC × TAG	2/91	3	19/18	78	8/15	53	1.46
ARO	2/91	3	19/18	78	8/15	53	1.46
GPT	2/91	3	13/21	62	8/15	53	1.16
MRD × TAC	2/91	3	18/21	86	8/15	53	1.61
SGD	2/91	3	12/19	63	8/15	53	1.18
CRD	2/91	3	11/20	55	8/15	53	1.03
BAN	7/91	2	10/12	83	16/26	62	1.35
BAO × CRD	7/91	2	22/29	76	16/26	62	1.23
BAY × SGD	7/91	2	23/29	79	16/26	62	1.29
CAT × RIT	7/91	2	13/19	69	16/26	62	1.11

Table 9.3. **(cont'd) Susceptibility of coconut populations screened for resistance to CCCVd using PAGE after mechanical inoculation.**

CAT × WAT	7/91	2	15/22	68	16/26	62	1.11
LAG × CRD	7/91	2	13/31	92	16/26	62	0.68
MYD × PYT	7/91	2	9/25	36	16/26	62	0.58
MVT	7/91	2	10/15	67	16/26	62	1.08
TAC × RIT	7/91	2	18/21	86	16/26	62	1.39
TAG × CRD	7/91	2	19/21	58	16/26	62	0.95
VIC	7/91	2	11/13	85	16/26	62	1.37
KAP	8/91	2	7/10	70	16/26	62	1.14

Table 9.4. **Variability of Tambolilid infection rate.**

Date of inoculation	No. assays	No. infected	% infected
7.86	7	66/70	94
1.87	7	30/30	100
4.88	7	23/40	58
8.88	6	30/40	75
5.89	6	19/20	95
6.89	5	7/16	44
7.89	5	7/17	41
1.91	3	19/20	95
1.91	3	15/16	94
2.91	3	8/15	53
7.91	2	16/26	62

Table 9.5. **Whole-crown PAGE assay of the 14 palms remaining negative for CCCVd after repeated inoculations with CCCVd.**

Population	Tree no	Total no. of fronds sampled	PAGE assay result (CCCVd forms)	Frond no. where CCCVd was detected
Alsua	740	27	negative	none
	916	13	246	0 and 1
	1100	17	negative	none
	1311	13	negative	none
CAT	3149	17	246, 247	0 to 7
MYD	2294	24	negative	none
	2873	13	negative	none
	3149	8	247	0 to 5
	4011	22	247	0 to 2
MRD	2354	17	negative	none
TAMB	6103	17	negative	none
	7211	24	negative	none
	7343	15	negative	none
		7361	negative	none

10 IN-VITRO CULTURE OF COCONUT EMBRYOS

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Introduction

The culture of embryos outside the seed was first performed by Hannig in 1904. It has since become a routine operation for many plants. Cutter and Wilson (1954) cultured normal coconut embryos in various media to elucidate the role of the endosperm during the post-embryonic growth of the embryo. They observed increases in size, but development of the shoot and root was very slow. In solid culture, the embryos germinated and produced several leaves, but formed only rudimentary roots (Abraham and Thomas 1962; de Guzman and del Rosario 1964).

De Guzman's team did an extensive study on the in-vitro culture of Makapuno coconut, whose embryo does not germinate in situ owing to abnormality of the endosperm. Their series of experiments showed that sequential culture from White's liquid (White and Davidson 1976) to Murashige and Skoog (MS) solid medium promoted concurrent and faster development of both shoot and root (Balaga and de Guzman 1971). Incorporation of activated charcoal in the MS solid medium and decapitation of roots of plantlets also improved root growth in embryo and seedling culture of Makapuno coconut (de Guzman and Manuel 1977). Del Rosario and de Guzman (1976) observed a promotive effect of high sugar content on root growth using MS medium. This effect could not be repeated when White's medium was used.

The Makapuno embryo culture technique developed by de Guzman and others found wider applications. Aside from rescuing the Makapuno embryo, in-vitro culture of coconut embryos facilitates germplasm collection, exchange and storage. It can also eliminate complicated phytosanitary treatment of heavy and bulky coconut seednuts.

Embryo culture is a useful tool in various research fields, such as in disease transmission studies (Orense and Pacumbaba 1988) and screening for resistance against cadang-cadang (Rillo et al. 1988b). More plantlets can be screened in a small space with the in-vitro system than with large and bulky coconut seednuts. The inoculation can also be done under controlled conditions.

Enlargement before germination of the embryos in White's liquid medium takes 6–8 weeks, which is rather long. Assy Bah (1986) used the elements from Murashige and Skoog (1962) as the basic medium and observed doubling of weight every 12–13 days, but did not indicate whether she tried other media formulations. The study described here in Chapter 10 was therefore aimed at evaluating the efficiency of these media formulations (White's, Murashige and Skoog, and Eeuwens' (1978) Y_3) in supporting growth and development of excised mature coconut embryos (9–11 months old).

The initial liquid culture of coconut embryos was shortened from 6 weeks on White's medium without activated charcoal (– AC) to 3 weeks in Eeuwens' Y_3 ± AC, and Murashige and Skoog (MS) + AC. The use of these media resulted in higher weight gain and percentage germination of embryos in a shorter time than with White's ± AC and MS – AC. Embryos grown in continuous darkness had higher weight gain than those grown in continuous light.

This Chapter describes the methods compared for the culture of coconut embryos at the ARC for the production of plants, as such plants are potentially useful for studies of cadang-cadang.

Methods

Nine- to 11-month-old embryos from Tambolilid seednuts were extracted using a No. 14 cork borer. The embryos, still enclosed in the solid endosperm, were initially disinfected with 100% commercial bleach (Chlorox®) for 30 min and then rinsed several times with sterile distilled water. The embryos were aseptically excised from the solid endosperm, again sterilised with 10% commercial bleach for 1–2 min, and then washed 3 times with sterile distilled water. The embryos were then blotted dry on sterile Petri dishes with filter paper. These operations were all done inside a laminar flow hood to avoid contamination.

Initial fresh weights of embryos were aseptically determined before culture. The average initial weight of embryos was 0.125 g. The following treatments were evaluated with 3 replications and 10 samples each:

- White's: + activated charcoal (AC), dark; + AC, light; – AC, dark; – AC, light
- MS (Murashige and Skoog): + AC, dark; + AC, light; – AC, dark; – AC, light
- Y_3 (Eeuwens): + AC, dark; + AC, light; – AC, dark; – AC, light.

Table 10.1 presents the nutrient composition of the 3 media formulations used. As activated charcoal in solid medium was observed to enhance growth of Makapuno embryos, its effect in liquid medium was also tested.

The embryos were cultured singly in 20 mm × 150 mm test tubes. These were kept in continuous darkness or in continuous light for comparison. For the continuous light treatment, 40 W fluorescent tubes were used. The incubation temperature for the cultures was 25°–27°C.

After 21 days, final fresh weights were aseptically taken, and percentage germination was recorded. An embryo was considered germinated when the shoot was about 1 mm long based on Eeuwens' (1978) criterion. After weighing, the embryos were transferred to MS solid medium with 4% sucrose (40 g/L), as recommended by del Rosario and de Guzman (1976).

Analysis of variance of the weight gained by the embryos was done using the 3-factor experiment in split-split-plot design. Duncan's Multiple Range Test was also applied.

Table 10.1. Nutrient composition of the 3 media formulations used.

Chemical Macroelements (mg/L)	Symbol	MS	Y3	White's
Ammonium nitrate	NH_4NO_3	1650	–	–
Ammonium chloride	NH_4Cl	–	535	–
Potassium nitrate	KNO_3	1900	2020	80.0
Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7\ H_2O$	370	247	720
Calcium chloride dihydrate	$CaCl_2 \cdot 2\ H_2O$	440	294	–
Potassium phosphate (monobasic)	KH_2PO_4	170	–	–
Potassium chloride	KCl	–	1492	65.0
Sodium phosphate (monobasic) monohydrate	$NaH_2PO_4 \cdot H_2O$	–	312	16.5
Sodium sulfate	Na_2SO_4	–	–	200
Calcium nitrate tetrahydrate	$Ca(NO_3)2 \cdot 4H_2O$	–	–	300
Microelements (mg/L)				
Potassium iodide	KI	0.83	8.3	0.75
Boric acid	H_3BO_3	6.20	3.10	1.50
Manganese sulfate tetrahydrate	$MnSO_4 \cdot 4H_2O$	15.6	11.2	5.28
Zinc sulfate heptahydrate	$ZnSO_4 \cdot 7H_2O$	8.60	7.20	3.00
Sodium molybdate dihydrate	$NaMoO_4 \cdot 2H_2O$	0.25	0.24	–
Cupric sulfate pentahydrate	$CuSO_4 \cdot 5H_2O$	0.025	0.16	0.10
Cobalt chloride hexahydrate	$CoCl_2 \cdot 6H_2O$	0.025	0.24	–
Nickel chloride hexahydrate	$NiCl_2 \cdot 6H_2O$	–	0.024	–
Molybdenum trioxide	MoO_3	–	–	0.01
Vitamins (mg/L)				
Meso–inositol		100	100	–
Nicotinic acid		5.0		5.0
Pyridoxine·HCl		0.50	0.05	0.50
Thiamine·HCl		0.50	0.50	0.05
Biotin		0.05	0.05	0.05
Folic acid		0.50	–	0.50
Glycine		2.0	–	2.0
Iron source (mg/L)				
Disodium EDTA	Na_2EDTA	37.3	–	37.3
Ferrous sulfate heptahydrate	$FeSO_4 \cdot 7H_2O$	27.8	–	27.8
Ferro-EDTA	FeEDTA		32.5	–
Sucrose (g/L)		30.0	45.0	20.0
Activated charcoal (g/L)		2.50	2.50	2.50

Results and Discussion

Analysis of the data showed that there was a highly significant correlation between the medium and the presence of 2.5 g/L of activated charcoal (Table 10.3). Table 10.2 shows that after 21 days in liquid culture, treatments Y3 + AC, Y3 – AC and MS + AC gave the highest weight gain of the embryos. These treatments were not significantly different at the 5% level. For Y3 medium, with or without AC growth of embryo was not significantly different. But there was a marked improvement of growth in MS medium when 2.5% AC was added.

Table 10.2. **Duncan's Multiple Range Test (DMRT) of mean weight gained by Tambolilid embryos in different media formulations after 21 days. Results were taken from samples grown in either light or darkness, as analysis of variance (Table 10.3) shows that there was no interaction between media and light conditions.**

Medium	Mean weight gained (g)*
MS + AC	0.677a
MS – AC	0.257b
White's + AC	0.228b
White's – AC	0.246b
Y3 + AC	0.725a
Y3 – AC	0.668a

* Means having a common letter are not significantly different at the 5% level.

Table 10.3. **Analysis of variance of weight gain of Tambolilid embryos grown in different media formulations and light conditions.**

				F test		
SV	**DF**	**SS**	**MS**	**FC**	**5%**	**1%**
Block/rep.	2	0.006471	0.003235	2.57996 ns	6.94	18.00
Media (A)	2	1.322750	0.661375	527.3686**	6.94	18.00
Error a	4	0.005016	0.001254			
+ AC/– AC (B)	1	0.193161	0.193161	68.4206**	5.99	13.74
A × B	2	0.340682	0.170341	60.3374**	5.14	10.92
Error b	6	0.016939	0.002823			
Light/dark (C)	1	0.134079	0.134079	14.5118**	4.75	9.33
A × C	2	0.025650	0.012825	1.3881 ns	3.88	6.93
B × C	1	0.005112	0.005112	0.5532 ns	4.75	9.33
A × B × C	2	0.018403	0.009202	0.99596 ns	3.88	6.93
Error c	12	0.110872	0.009239			
TOTAL	35	2.179135				

c.v. (A) = 7.5%; c.v. (B) = 11.30%; c.v. (C) = 20.45%.

** = significant at 1% level; ns = not significant.

De Guzman et al. (1971) observed that, after 8 weeks in White's liquid medium, Makapuno embryos increased in size but generally remained ungerminated until after their transplantation to solid medium. Balaga and de Guzman (1971) had concluded that 6 weeks in White's liquid culture appeared to be the minimum period for optimum root growth and for maximum shoot growth of Makapuno when the embryo was subsequently transferred to MS solid medium with 2.5 g/L activated charcoal. Such observations were true also for embryos from normal nuts using White's liquid medium. However, in our study, there was a rapid enlargement of embryos in Y_3 + AC, Y_3 – AC and MS + AC media within 21 days (Table 10.4). In some cases, germination of plumule or roots had even begun in liquid medium (Table 10.4). Some embryos that were not germinated already had protrusions on the apical portion where the plumule was expected to germinate, and some showed darkening at the site where the radicle would eventually grow. Germination of embryos or early signs of germination while still in liquid culture indicats that a high proportion of germinated embryos will result from transfer to solid medium (Table 10.4).

Table 10.4. Percentage germination of Tambolilid plantlets cultured initally in liquid and subsequently on MS solid medium.

Media	After 21 days in liquid	After 36 days on solid
MS + AC	43	83
MS – AC	10	50
White's + AC	0	50
White's – AC	0	30
Y_3 + AC	30	77
Y_3 – AC	23	73

Embryos from White's + AC and White's – AC started to germinate 36 days after transfer onto MS solid medium, whereas those in MS + AC, Y_3 + AC and Y_3 – AC were already fully germinated. That is, the plumule was more than 1 mm long with 2 scale leaves, and the first root was already well defined. This stage was attainable in White's medium only after 8 weeks or more on solid medium. Table 10.4 shows that embryos initially cultured in MS + AC liquid medium gave the highest percentage of germination when subsequently cultured on MS solid medium.

Del Rosario and de Guzman (1976) suggested that, for any given sugar level at transplantation, increasing sugar content during the initial culture may give favourable effects. Thus it was not surprising that greater weight gain and percentage germination were obtained in embryos initially cultured in MS + AC and Y_3 ± AC. These media contain higher concentrations of mineral nutrients and sugar (Table 10.1).

The effect of light on in-vitro cultures is one of the many areas not yet thoroughly evaluated. De Guzman (1970) investigated the effect of light on the development of germinated embryos in liquid culture. She noted that light did not inhibit the development of the root and that satisfactory results were obtained under a variety of conditions. She did not report, however, what effect light has on the weight gain of embryos in the initial liquid culture. Our study has shown significantly greater weight gain of embryos under continuous darkness than under continuous light (Table 10.5). A parallel can be drawn with in-situ germination of seednuts, where light is excluded.

Table 10.5. Duncan's Multiple Range Test of mean weight gained by coconut embryos cultured under continuous dark or light conditions.

Culture conditions	Mean weight gained*
Dark	0.531a
Light	0.409b

* Means having a common letter are not significantly different at 5% level.

Conclusions

Y_3 + AC, Y_3 – AC and MS + AC media are recommended for the initial liquid culture of embryos. These media resulted in higher weight gain and percentage germination of embryos in a shorter time than White's ± AC and MS – AC. There was also a higher weight gain of embryos grown in continuous darkness than in continuous light. The liquid culture period of Tambolilid embryos was shortened from 6 weeks in White's – AC to 3 weeks in any of the media recommended above.

11 SCREENING OF COCONUT POPULATIONS FOR RESISTANCE TO CADANG-CADANG DISEASE USING IN-VITRO CULTURED PLANTLETS

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Introduction

Identification of resistant individuals and breeding for resistance is generally considered for the control of pathogens when other control measures are not available. Evaluation of resistance to CCCVd using mechanical inoculation procedures has obvious advantages over field resistance studies because the screening time is reduced to a few years. With mechanical inoculation the infection pressure can be increased by adjusting the amount of viroid injected. The inoculation method uses high-pressure injection to force RNA extracts into coconut sprouts (Rodriguez et al. 1987, and see Chapter 9).

The development of an effective, aseptic inoculation technique for coconut plantlets grown in vitro could be useful for understanding the transmission and physiology of the disease, and for the screening of sterile material for resistance. The tissue culture techniques also allow economies of space, time and labour. An in-vitro, sterile, mechanical inoculation technique for coconut plantlets in tissue culture was therefore developed for resistance screening. Results from this work should complement the results of resistance trials using emerging sprouts in whole nuts.

Several methods of aseptically inoculating plantlets grown in vitro with the viroid have been tried previously, such as immersion of decapitated roots of the plantlets in the inoculum, injection of inoculum using a hypodermic needle, and blade slashing. Only slashing in combination with high-pressure injection has been used successfully to transmit CCCVd, and so the blade slashing technique was used for the resistance screening trials described here (Rillo and Paloma 1989).

This chapter also describes a trial done to determine the most effective concentration for inoculation in vitro, and another trial set up to determine the incubation period required to achieve the maximum infection rate.

Methods

Culture of embryos

Tambolilid embryos (8–10 months old), still completely enclosed by the solid endosperm, were extracted from seednuts as described in Chapter 10. The embryos were then transferred singly to tubes containing Murashige and Skoog (MS) liquid medium with 2.5 g/L activated charcoal (AC). After 3–4 weeks' incubation, the embryos were transferred to MS solid medium, also with 2.5 g/L AC but with a higher sucrose level, and then allowed to grow for 8 weeks, after which they were ready for inoculation. They were removed from the tubes and washed with

sterile distilled water to remove particles of agar and activated charcoal clinging to the base of the plantlets, then blotted dry.

Preparation of inoculum

Inoculum was supplied by the Virology-Biochemistry Division at PCA-ARC at a concentration of 20 μg/mL. This was found to be the optimum concentration in a study to determine the factors affecting transmission to coconut seedlings (Imperial et al. 1986). The CCCVd inoculum was sterilised by filtration through 0.45 μm, then 0.22 μm nitrocellulose membrane filters. Three concentrations of CCCVd (20 μg/mL, 1.5 μg/mL and 0.13 μg/mL) were used to determine the most effective concentration for inoculation in vitro.

Inoculation

The CCCVd inoculum was poured into a sterile petri dish. The coconut plantlets were immersed in the inoculum and several blade slashes (10–20, depending on the size) were made around the base. Inoculated plantlets were transferred to fresh medium with 2.5 g/L AC and incubated in the screenhouse until assayed. Detection of the viroid in the plantlets was by polyacrylamide gel electrophoresis (PAGE) and molecular hybridisation assay (MHA).

Collection of large numbers of embryos

Tambolilid populations were collected from marked palms at ZRC, in Calasgasan, Camarines Norte. The trees had been previously assayed and shown to be free of detectable viroid. A collection method was developed (E. Rillo, unpublished) to facilitate the transport of large numbers of embryos from ZRC. This method would make it possible to transport embryos from any point in the world and could thus facilitate coconut germplasm exchange.

Nuts were husked and split into halves, and the endosperm cylinders containing the embryos were collected with a punch using a No. 10 cork borer. Pre-disinfection was done at ZRC by soaking the endosperm cylinders enclosing the embryos in 100% commercial bleach for 30 minutes and then washing several times with sterile distilled water. They were sealed in sterilised plastic bags lined with cotton soaked in distilled water. These disinfection operations could be done in a simple isolation box in a room with still air to minimise contamination. The embryos were transported in cold storage in an ice chest.

Upon reaching the laboratory, the cylinders were disinfected again in 100% commercial bleach for 5 minutes and then washed several times with sterile distilled water. The embryos were aseptically excised from the endosperm using a scalpel and forceps in a sterile petri dish lined with filter paper. Subsequent sterilisation, culture and inoculation followed the procedure described in Chapter 10.

After inoculation, the plantlets were maintained in the screenhouse at a temperature of 30°–35°C until they were ready for assay.

Results and Discussion

Table 11.1 shows that, even with an inoculum concentration of 0.13 μg/mL, the viroid was transmissible in vitro and was detected by MHA. However, to obtain maximum infectivity, a 20 μg/mL concentration was used for the mechanical inoculation of in vitro-grown plantlets. Usually, about 100 plantlets could be inoculated with 40 mL of inoculum.

Table 11.2 summarises the preliminary results of the trial to determine the incubation period required to achieve the maximum infection rate using Tambolilid embryos. The viroid was detected in the plantlets by MHA 22 and 23 days after inoculation. Although it is possible that the CCCVd detected was residue from the inoculum, the tissue assayed was from the leaf and not from the area where slashing took place. Table 11.2 also shows that, after 3 months' incubation, 4 out of 12 of the plantlets tested by PAGE were positive.

With these findings, inoculation trials were expanded to include different coconut populations collected from Zamboanga Research Center (ZRC), which is in a cadang-cadang free area. Table 11.3 summarises the total number of coconut cultivars and hybrids collected from ZRC inoculated and assayed. Table 11.4 shows the preliminary results of periodic assays of different coconut cultivars inoculated with 20 μg/mL of CCCVd. The data show that results from the first assay could not be repeated and the viroid could rarely be detected even by the more sensitive MHA, which can detect the viroid after 1 month of incubation. It is therefore possible that viroid detected in the first assay was residual inoculum.

PAGE assays yielded negative results except for 2 CAT × LAG out of 6 plantlets, 1 MRD × BAY out of 6 plantlets, and 1 Zamboanga Tall out of 17 plantlets submitted for assay 6 months after incubation. Thus it was decided that 6 months should be the optimum incubation period before the inoculated plantlets were assayed.

One advantage of extending the incubation period to 6 months is that the test plantlets would have formed enough true leaves that could be used for assay. Positive presence of the viroid from the leaf extracts would indicate that the viroid had replicated and translocated from the point of inoculation to the leaves. This makes a non-destructive assay of the plantlets possible, although we found it very difficult to keep the plantlets sterile throughout the incubation period. If the plantlet tested negative for the presence of the viroid after 6 months' incubation, it would be possible to return to that plantlet to determine whether, at a later date, it will be found positive for the presence of the viroid. These features will be helpful in the screening for resistance in coconut cultivars to CCCVd using in-vitro-grown plantlets.

Table 11.1. MHA results of Tambolilid seedlings inoculated with 3 concentrations of CCCVd.

Concentration (μg/mL)	No. seedlings inoculated	Results (positive/total)	
		Na_2SO_3 –PEG	Na_2SO_3–TCA
20	25	PAGE results gave weak positives	
1.6	53	2/52	3/53
0.13	52	2/52	4/52

Table 11.2. Results of periodic assay for CCCVd in Tambolilid seedlings mechanically inoculated in-vitro with an inoculum concentration of 20 μg/mL.

Incubation period	Assay results (positive/total)	
	PAGE	MHA
22 days	no assay	4/4
23 days	no assay	8/15
1 month	1/2	no assay
2 months	0/10	no assay
3 months	4/12	no assay

Table 11.3. Coconut cultivars and hybrids from ZRC inoculated and assayed.

Cultivars	Number of plantlets	
	Inoculated	Assayed
Makilala	170	163
Catigan	54	49
Tambolilid	435	403
Baybay	198	180
Laguna	164	153
Cat × Lag	231	208
MYD	129	113
Magtuod	11	11
MRD × BAY	192	168
Tacunan	70	62
TAC × WAT	91	74
MYD × WAT (MAWA)	104	84
Tagnanan	53	51
Rennell	43	27
Bao	44	37
WAT	21	19
Tahiti	45	45
Hijo Tall (TRRC)	15	10
Zamboanga Tall	68	65
Kinabalan Dwarf	64	60
OD	16	13

Table 11.3. (cont'd) Coconut cultivars and hybrids from ZRC inoculated and assayed.

HGT (TRRC)	20	7
MRD × HGT (TRRC)	21	21
EGD	60	57
Spikata	32	31
Pilipog	31	27
YD	19	11
San Ramon	50	38
Karkar	45	43
TOTAL	2496	2230

Table 11.4. Number of positive plants out of total number tested in periodic assay (MHA or PAGE) of different coconut cultivars inoculated with 20 μg/mL of CCCVd.

Cultivars	**Incubation period (months)**								**Total no. plantlets tested**
	MHA							**PAGE**	
	1	2	3	4	5	6	7	6	
TAM	5/49	0/46	3/34	0/9					138
CAT	11/30	0/17	0/2						49
MAK	4/44	1/38	0/25	0/10	0/16				145
BAY	12/60	0/47	0/35	0/19	0/5	0/5			171
LAG	0/32	0/28	0/20	0/10	0/5	0/5			101
CAT × LAG	1/51	0/44	1/31	0/15	0/26		0/5	2/6	172
MYD	9/36	0/18	0/11	0/3					68
MAG	2/11								11
MRD × BAY	3/45	1/40	0/26	0/18	0/3	0/5		1/6	137
TAC	3/29	0/19	0/10	0/2					60
TAC × WAT	1/43	1/20	0/7	0/2	0/2				74
MAWA	1/20	0/11	0/13	0/4		0/3			51
TAG	0/9	0/5							14
TAH	0/6	0/5							11
BAO	0/5	0/5							10
HGT (TRRC)	0/5								5
Zamboanga Tall	0/5	1/12						1/17	17
EGD	1/14								14
KIN	1/5	0/5							10
RNL	0/7	0/5							12
OD	0/5	0/4							9
Hijo Tall	0/5								5
TOTAL									1284

12 IN-VITRO INOCULATION OF EMBRYOS FOR RESISTANCE SCREENING, AND ASSAY OF EMBRYOS CULTURED FROM DISEASED PALMS

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Introduction

In the absence of direct control measures for CCCVd, screening of different coconut cultivars to identify resistant individuals may provide a possible means of control. Mechanical inoculation of 0–7-day-old sprouts of germinating nuts using a high-pressure injector is used routinely for resistance screening of cultivars (Imperial et al. 1985). The method has the disadvantage, however, of requiring considerable space. This chapter describes a reproducible system for inoculating in-vitro-germinated seedlings with viroid, with potential for use in controlled studies of CCCVd pathogenicity. Tissue culture also removes the risk of accidental natural infection of coconut tissue.

Methods

Collection and culture of embryos

Tambolilid palms were assayed by polyacrylamide gel electrophoresis (PAGE) to identify CCCVd-free sources of nuts. Ten- to 11-month-old nuts were harvested and their embryos were extracted and disinfected using the procedure of Rillo and Paloma (1990). Excised embryos were inoculated singly in test tubes containing liquid Murashige and Skoog (MS) medium supplemented with 2.5 mg/L of activated charcoal. After 3–4 weeks of incubation, they were subcultured to solid MS medium with a higher level of sucrose and incubated at room temperature (as described in Chapter 11).

Preparation of inoculum

Inoculum was prepared using the procedure developed by Imperial et al. (1985). The 4 forms of CCCVd were present in the inoculum.

Inoculation

CCCVd inoculum in 0.1 × SSC (1 × is 0.15 M NaCl, 0.015 M Na citrate) was filter-sterilised successively through 0.45 μm and 0.22 μm nitrocellulose filters (Millipore®) and checked for RNA content by PAGE assay. Three to four weeks after germination, the embryolings were inoculated by either blade slashing or high-pressure Panjet injection. For blade slashing, the germinated embryos (white) were slashed 5–8 times while immersed in liquid MS medium and

then transferred to fresh, solid MS medium, and the CCCVd inoculum of 9 μg/mL was applied aseptically. For the high-pressure Panjet inoculation, each embryo was given three 100 μL injections with a total of 9 μg CCCVd before being transferred to fresh solid medium. Inoculated cultures were incubated at room temperature with an 8–10 h day-length. Uninoculated germinated embryos and embryos mock-inoculated with 0.1 × SSC served as controls.

Assay

Control and inoculated embryolings were assayed 6 months after inoculation. Leaf tissue (0.5–1 g) was sampled and nucleic acids were extracted using the PEM bag method (see Chapter 3). Nucleic acid extracts were assayed by either PAGE or dot-blot hybridisation assay (MHA). Subsequent assays were done at 12 and 18 month.

Maintenance of cultures

Cultures were transferred routinely every 4–8 weeks to fresh, solid MS medium.

Results and Discussion

Inoculation of embryos in vitro

Neither of the two inoculation methods led to the detection of CCCVd in leaflets of the cultured embryos at 6, 12 or 18 months. The survival rate of embryolings was between 8% and 44% at 6 months, between 0.3% and 17% at 12 months, and between 0% and 3% at 18 months (Table 12.1). Because survival rates were poor overall, it is not possible to distinguish whether the inoculations were unsuccessful or the inoculated embryos did not survive.

Assay of embryos from infected symptomatic palms

To determine whether embryos collected from nuts of diseased palms contained detectable levels of CCCVd when cultured in vitro, 3 sites were selected in cadang-cadang areas.

A total of 434 nuts were collected from 32 diseased source trees belonging to 6 varieties (1 tall, 1 dwarf, 4 hybrids). Of these, 21 contained the 247-nucleotide form of CCCVd, 9 contained the 246 form or a mixture of the 246 and 247 forms, and 2 contained the 296 nucleotide form. Of the isolated embryos, 262 germinated, representing 60% of the nuts. Of these 262, 120 embryolings or plantlets (46%) were tested for CCCVd. All were negative in the first assay. No follow-up assay was performed as the project was terminated.

Acknowledgments

We gratefully acknowledge the support provided by the Philippine Coconut Authority and the Australian Centre for International Agricultural Research (ACIAR) for support. This work forms part of the PCA-ACIAR Project No. 8402.

Table 12.1. Summary of inoculation experiments with coconut embryos cultured in vitro.

Treatment	Inoculum	No. embryos	Showing formation of: Shootroots				Surviving and assayed after 6 months by: MHA PAGE				Surviving and assayed after 12 months by: MHA PAGE				Surviving and assayed after 18 months by: MHA PAGE				Incidence of CCCVd infection by PAGE among survivors at 6 months
			no.	%	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%	
uninoculated	–	112	98	88	71	63	33	29	22	20	17	15	4	4	2	2	0	–	< 2%
slash inoculation	CCCVd buffer	276	238	86	145	53	110	40	39	14	46	17	5	2	4	1.5	0	–	< 0.7%
		111	98	88	81	73	34	31	28	25	9	8	7	6	1	1	0	–	< 1.6%
Panjet injection	CCCVd buffer	309	261	84	189	61	137	44	30	8	45	15	1	0.3	8	3	0	–	< 0.6%
		115	106	92	74	64	38	33	29	25	17	15	1	1	2	2	0	–	< 1.5%

13 BROOMING, A SEVERE FORM OF CADANG-CADANG DISEASE

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Introduction

The naturally occurring sequence variants of CCCVd reported so far have been discussed in the previous chapters. Variants that arise during disease progression all contain the basic 246 nucleotides. The difference between variants 246 and 247 and between 296 and 297 is the addition of only one cytosine residue at position 197 (Haseloff et al. 1982). This variation can be detected by the non-denaturing polyacrylamide gel electrophoresis procedure described by Imperial and Rodriguez (1983). With this procedure for routine diagnosis of CCCVd in coconut palms, minor bands that had not been described previously were observed between bands of the four monomeric CCCVd forms in some cadang-cadang infected palms with particularly severe symptoms. This chapter describes the relationship between the presence of these bands and the severity of symptoms of the disease.

Observation of a Severe 'Brooming' Symptom in CCCVd Inoculated Palms

At the Albay Research Center (ARC) of the Philippine Coconut Authority (PCA), Philippines, inoculation with CCCVd preparations is done with a hand-primed, high-pressure injector. Since the small variants of CCCVd are more infectious than the larger variants (Mohamed et al. 1985), the inoculum is prepared from a mixture of the 246 and 247 variants, as determined by their mobility in the gel relative to the CCCVd standard marker. The inoculum is prepared in batches from leaf material obtained from various palms in local coconut plantations or at the ARC. Preparations of inoculum are stored in ethanol at –20°C and remain infective for at least 3 months (Randles et al. 1992). Approximately 1 μg of CCCVd is inoculated into each test palm.

For the early inoculations shown in Table 13.1, yearly monitoring of infection by PAGE assay and observation for symptoms revealed varied reactions to inoculation. The rate of transmission, regardless of the coconut population, was higher when sprouts were inoculated at 1–7 days after emergence rather than when the sprouts were 2–3 months old. In addition, the rate of symptom development varied. An obvious new symptom type appeared in a significant number of palms. It was characterised mainly by loss of leaf lamina, giving the palm a 'broomed' appearance associated with severe stunting of the palm (Figure 13.1). The basal fibres also remained attached to the fronds as in a typical cadang-cadang infected palm. The observation of this severe symptom was not specific to a particular coconut population (Table 13.1). For the 2–3-month-old inoculated palms, the brooming symptom first appeared 4–7 years after inoculation; for the 1–7-day old inoculated sprouts, the symptom first appeared 3–5 years after inoculation. The palms inoculated in 1981 and 1982 had a higher brooming incidence (18.8% and 22.7%, respectively) than the palms inoculated in 1983–1986 (4.4%–12.9%). Premature death was observed in some of the palms. Although there is no direct evidence that death was caused by the severity of the disease, this observation is unusual even for palms with the typical cadang-cadang symptoms.

Table 13.2 shows the results of an infectivity trial in which the small and large variants of CCCVd, determined by their mobility in the gel, were isolated from each other and tested for their ability to infect. Tenfold dilutions of each variant were inoculated; palms received inocula in the range of 200 ng per palm to 200 pg per palm. The rate of transmission was higher in palms inoculated with the 246 and 247 variants than in the palms inoculated with the 296 and 297 variants. Brooming incidence was essentially confined to palms inoculated with the 246 or 247 variant; only one case of a brooming palm occurred among those inoculated with the 296 variant.

In all of the trials conducted, a total of 1787 successfully inoculated palms have been monitored; 12% of these had brooming symptoms.

Figure 13.1. **The unusual, severe brooming symptoms observed in coconuts inoculated with CCCVd variants 246 and 247.**

Figure 13.1a. **The palm is severely stunted and has a 'broomed' appearance owing to the reduction of the lamina. The basal fibres remain attached to the fronds as in a typical cadang-cadang-infected palm.**

Figure 13.1b. **The frond is shorter (left) than a healthy frond (right).**

Figure 13.1c. **Leaflets have a smaller lamina (left) than cadang-cadang-infected leaflets (middle) and healthy leaflets (right).**

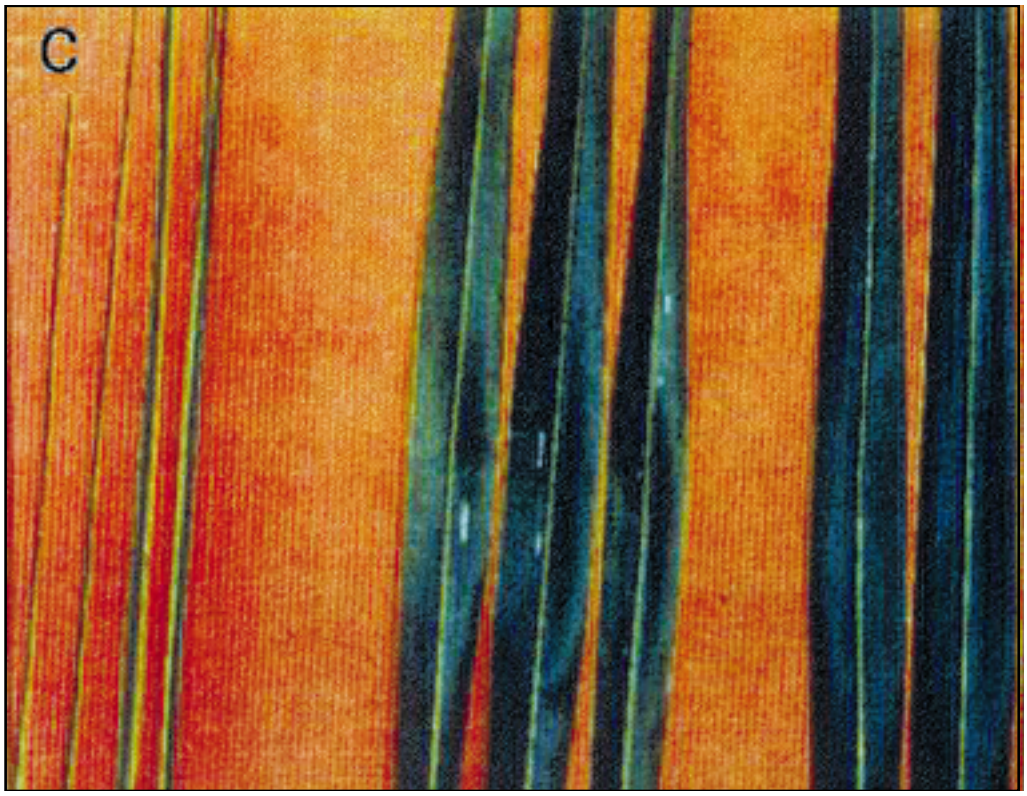


Table 13.1. Incidence of the brooming symptom in various coconut populations inoculated with CCCVd isolated from palms with 246 and 247 forms only.

Inoculation			PAGE assay		Appearance of brooming symptom		
Year	Age of test palms (p.e.)*	Population inoculated	Years after inoculation	No. positive/no. inoculated	Years after inoculation	No. with brooming	% brooming
1981	2–3 mo	Laguna	6	22/150	6	2/22	19.7
		Baguer	6	1/30	7	1/1	
		Banga	6	1/30	–	0/1	
		Spicata	6	9/30	6	4/9	
		Gatusan	6	19/30	6	4/19	
		Orange Dwarf (OD) × Baybay	6	11/30	–	0/11	
		Catigan × Rennel	6	5/30	6	3/5	
		Tacunan × West African Tall (WAT)	6	8/30	6	1/8	
1982	2 mo	Malayan Yellow Dwarf (MYD)	5	1/17	–	0/1	22.7
		Tambolilid	5	7/73	5	3/7	
		Catigan	5	3/29	–	0/3	
		Magtuod	5	4/30	5	2/4	
		Cameroon Red Dwarf (CRD)	5	2/18	–	0/2	
		Kapatagan	5	5/29	–	0/5	

Table 13.1. **(cont'd) Incidence of the brooming symptom in various coconut populations inoculated with CCCVd isolated from palms with 246 and 247 forms only.**

1983	2 mo	mixed populations	4	300/750	4	18/300	5.9
		MYD × WAT	4	3/24	–	0/3	
		OD × WAT	4	3/24	–	0/3	
1983	1–7 d	MYD × WAT	4	11/24	–	0/11	12.9
		STB† × STP†	4	14/24	5	4/14	
		CRD × CRD	4	15/22	5	2/15	
		STP† × STP†	4	21/24	5	5/21	
		YD† × LT†	4	21/24	5	4/21	
		OD × WAT	4	15/24	–	0/15	
		STB × STB	4	18/24	–	0/18	
		CRD × LT	4	13/23	5	1/13	
		OD × LT	4	19/24	5	3/19	
1985	1–7 d	Tambolilid	2	286/550	3	17/286	4.4
		Catigan	2	104/275	3	1/104	
		OD	2	135/280	–	0/135	
		MYD	2	114/270	–	0/114	
		Tambolilid	2	90/174	4	14/90	
1986	1–7 d	Tambolilid	1	242/450	4	17/242	7.0

* Post-emergence
†STB, STP, LT and YD are local Philippine selections

Table 13.2. Incidence of the brooming symptom in Tambolilid palms inoculated 1–7 d after emergence with purified CCCVd.

Inoculation		PAGE assay			Incidence of brooming	
Year	Form of CCCVd	Years after inoculation	No. positive/no. inoculated	Years after inoculation	No. with brooming	% brooming
1982	247	5	38/44	5	5/38	14.3
	246	5	22/28	5	4/22	
	247+246	5	10/12	5	1/10	
1982	297	5	18/28	5	0/18	2.7
	296	5	19/28	5	1/19	
1983	247	4	30/38	5	10/30	20.0
	246	4	34/43	5	7/34	
	247+246	4	41/56	5	4/41	
1983	297	4	22/35	5	0/22	0
	296	4	15/33	5	0/15	
	297+296	4	16/43	5	0/16	

Correlation of the Brooming Symptom with Distinct Electrophoretic Forms of CCCVd

The PAGE assay of palms with brooming symptoms was done as described in Chapter 2. The leaf material, however, was obtained from successive or alternate fronds within the whole crown of each palm (Imperial and Rodriguez 1983). Thus, an average of 12 fronds were individually assayed for each palm following the first observation of the brooming symptom. This was followed by another whole-crown assay approximately a year after. Assays before the appearance of the brooming symptoms were done on the second- or third-youngest open frond. The whole-crown assay is necessary to determine the overall viroid pattern of the palm as there may be differences in pattern in some fronds associated with the development of the disease (Imperial et al. 1981; Imperial and Rodriguez 1983).

A total of 136 palms with brooming symptoms were screened; the viroid patterns obtained are summarised in Table 13.3. All the palms were found to have bands other than the CCCVd variants previously described as 246, 247, 296 or 297 (Haseloff et al. 1982). The latter were detected in palms with typical cadang cadang symptoms, in agreement with the previous data (Imperial and Rodriguez 1983). A total of 11 extra bands were observed; these were letter-coded according to their position in the gel relative to the CCCVd marker. Three of these bands were between CCCVd variants 247 and 246; 2 were between 246 and 297; 3 were between 297 and 296; and 3 were above 296.

Figure 13.2 shows examples of viroid patterns from palms with varying intensities of the brooming symptoms. The distinct forms fractionated in 20% non-denaturing PAGE were visualised by silver staining (Figure 13.2a) as described in Chapter 2, and by hybridisation to a ^{32}P-labelled cDNA probe (Figure 13.2b) as described in Chapter 2. Their circularity was confirmed by 2-D PAGE (Chapter 2), in which a 20% non-denaturing gel was used in the first dimension and a 10% denaturing gel was used in the second dimension (Figure 13.2c). These viroid forms could not be detected in 5% and 10% non-denaturing PAGE, which suggests that there were only minor differences in their nucleotide sequences compared with the common CCCVd forms. These results indicate that the electrophoretic forms associated with the severe brooming symptoms differed in primary and secondary structure from CCCVd, and are probably mutants of CCCVd.

Figure 13.2. **Assay of palms with varying intensities of the brooming symptoms.**
Figure 13.2a. **20% non-denaturing PAGE assay of extracts from palms (nos 1–5; see Table 13.4) and a mixture of their isolates in lane no. 6. The bands were visualised by silver staining.**

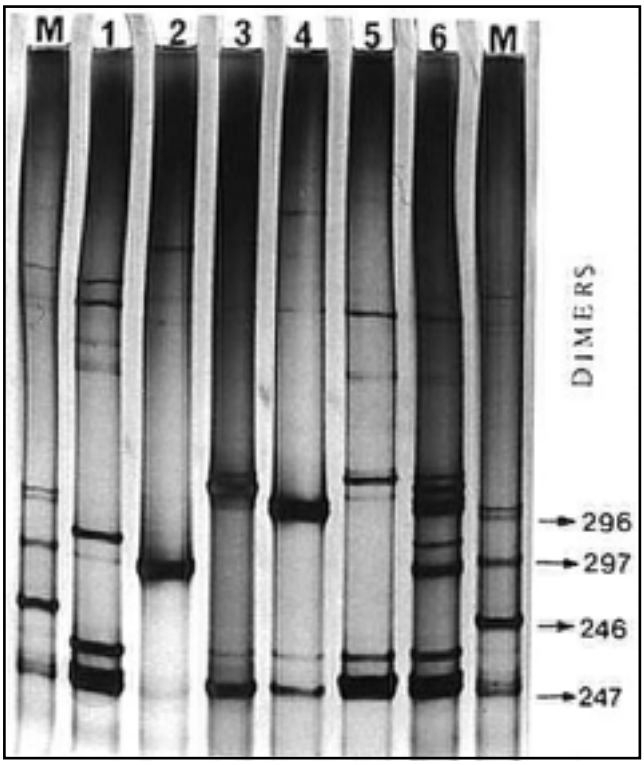


Figure 13.2b. **Hybridisation assay of an electroblot from a 20% non-denaturing PAGE of the samples in Fig. 13.2a.**

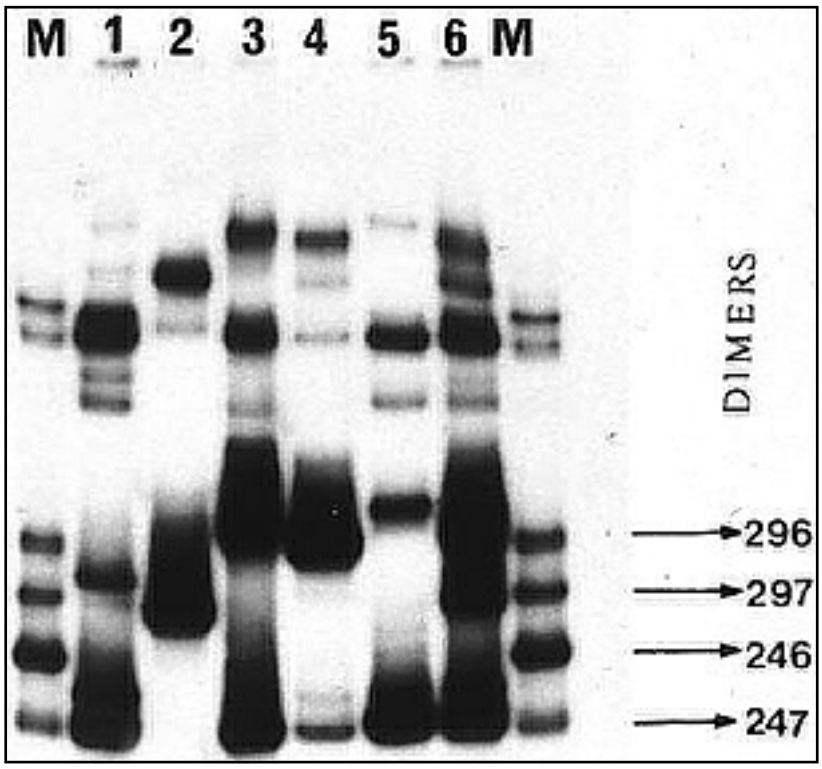
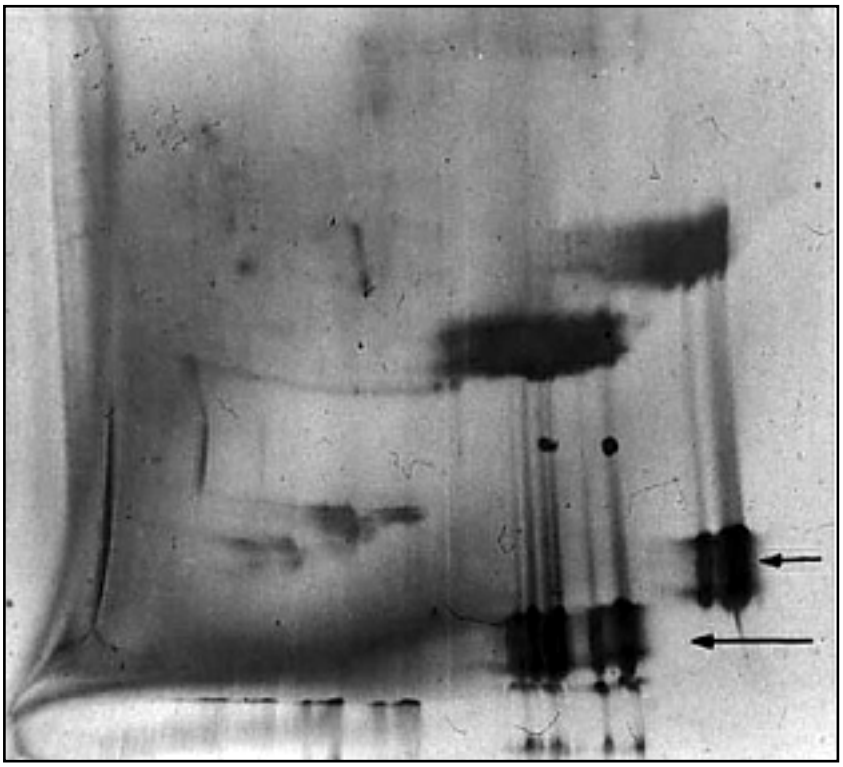


Figure 13.2c. **Two-dimensional PAGE of a mixed isolate (lane no. 6 in Figs 13.2a and b). Assay used a 20% non-denaturing gel in the first dimension and a 10% denaturing gel in the second dimension. The arrows show the position of circular viroid molecules.**



Variation in the Viroid Patterns during Disease Development

Twelve palms were analysed for their viroid patterns from the time the viroids were first detected until the brooming symptom appeared (Table 13.4). The final PAGE assays of 5 of these palms are shown in Figure 13.2. Infection started almost always with variant 247, although the inoculum was a mixture of variants 246 and 247. Then, approximately a year before brooming appeared, additional bands with varying gel mobility were detected. As the disease developed, more variations were observed, the size of the molecules increasing. However, there were at least 2 palms (with viroid patterns 2 and 3, Table 13.3) that died before the larger molecular variants could be detected. But again, there was no direct proof that the death was caused by infection with the observed electrophoretic forms associated with brooming symptoms.

Table 13.3. **Viroid patterns in 20% non-denaturing PAGE (whole-crown assay) of palms with the brooming symptom shown in relation to frequency of detection.**

Pattern no.	Monomer region*															No. palms screened
	296c	296b	296a	296	297c	297b	297a	297	246b	246a	246	247c	247b	247a	247	
1														•	•	6
2													•		•	16
3												•	•		•	1
4													•	•		2
5												•		•		8
6													•			6
7												•				2
8										•	•		•			1
9									•						•	3
10									•				•		•	2
11								•					•		•	6
12						•		•					•		•	2
13						•							•		•	5
14				•	•								•		•	4
15			•										•		•	2
16		•	•	•									•		•	3
17							•	•					•	•	•	3
18				•							•		•		•	1
19	•	•		•									•	•	•	2
20	•					•							•		•	1
21						•							•	•		2
22	•	•											•	•		1
23								•					•			3
24						•		•					•			1

Table 13.3. **(cont'd) Viroid patterns in 20% non-denaturing PAGE (whole-crown assay) of palms with the brooming symptom shown in relation to frequency of detection.**

25						•							•		1
26				•		•							•		2
27				•									•		4
28			•										•		2
29	•												•		3
30				•				•					•		1
31						•	•						•		1
32								•				•		•	2
33			•		•							•		•	2
34						•						•		•	2
35			•			•						•		•	2
36			•									•		•	2
37	•											•		•	5
38						•					•		•		1
39			•			•							•		3
40						•		•							3
41						•									8
42				•		•									3
43	•	•	•	•		•		•							1
44	•					•									1
45			•	•											1
46	•	•		•											1
47							•								2
Total															136

* Positions of the electrophoretic forms designated a, b and c are shown relative to the marker CCCVd$^{247\ 246\ 297\ 296}$.

Table 13.4. Time-course analysis of viroid pattern before the appearance of brooming.

Palm sample	Year inoculated	Year brooming observed	Viroid pattern							Pattern no. in Table 13.3	Isolate no. in Fig. 13.2
			1984	1985	1986	1987	1988	1989	1990		
Magtuod 1	1982	1987	247	NA*	247, 247a	247, 247a, 247b, 297, 297a				17	1
STB × STP 1	1983	1988	NA	247	247	247, 246b	247, 246b			9	2
OD × LT 1	1983	1988	247	NA	247	247, 247b	247, 247b, 296, 296a, 296b			16	3
STP × STP 1	1983	1988	–	NA	247, 246	NA	247, 247b, 297c, 296			14	4
STP × STP 2	1983	1988	247	NA	247, 247b	NA	247, 247a, 247b, 296, 296b, 296c			19	5
Tambolilid no. 1	1985	1988			–	247, 246	247b, 246, 246a			8	
Tambolilid no. 2	1985	1989			NA	247	NA	247a, 247b		4	
Tambolilid no. 3	1985	1989			+DB†	NA	247a, 247b	247a, 247b		4	
Tambolilid no. 4	1985	1989			NA	247	NA	247b		6	
Tambolilid no. 5	1985	1989			247	NA	247, 247b	247, 247b		2	
Tambolilid no. 6	1985	1989			247	247	247, 247b	247b		6	
Tambolilid no. 7	1986	1990				+DB	+DB	247b	247, 247b	2	

* NA = not assayed
†+DB = positive in dot-blot assay

Discussion

This paper describes the spasmodic appearance of a previously undescribed, unusually severe symptom following inoculation of seedlings with CCCVd. The leaf lamina is so greatly reduced that the remaining midribs resemble the bristles of a broom. The name given to this severe form of disease is brooming. Brooming is not uncommon, as it was observed in approximately 12% of the 1787 CCCVd-inoculated palms screened. Observations suggest that palms with this symptom have a shorter life than those that show the more common mild yellowing, sterility and stunting. It may therefore have a higher incidence than reported because of this higher mortality. It was normally first observed 3–7 years after inoculation, and it was found almost exclusively in palms inoculated with either the 246 or 247 variants of CCCVd. Analysis of the viroid forms in 136 palms with brooming by PAGE and hybridisation showed that all of them contained electrophoretic forms distinct from the previously described 246, 247, 296 and 297 nucleotide variants of CCCVd. The use of non denaturing 20% PAGE has previously been shown to identify variants of CCCVd, presumably because changes in secondary structure that are associated with nucleotide changes markedly affect their electrophoretic mobility. There were 11 additional bands found by PAGE in association with brooming; this suggests that a number of mutations are associated with the syndrome. Sequencing of a number of these variants (Rodriguez and Randles 1993) has shown that the mutations associated with brooming occur at 2 or 3 sites in the molecule, and may be deletions, additions or substitutions of nucleotides. A next step would be to produce full-length clones of these mutant forms and to inoculate them separately to coconut to observe their pathogenicity.

One of the important consequences of this study is that it shows that CCCVd is mutable, and that severe mutants can exist in palms growing in the field. Occasional observations of similar palms in plantations suggest that the brooming occurs naturally and that CCCVd diversity could be monitored effectively by the 20% non-denaturing PAGE assay. Recognition of this form of the disease indicates that mutation may lead to a range of disease symptoms in cadang-cadang areas, and that molecular procedures to monitor mutation frequencies could be introduced in future to improve the understanding of the disease.

14 EVIDENCE THAT SYMPTOM EXPRESSION OF CADANG-CADANG DISEASE MAY BE INFLUENCED BY ALTITUDE

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Rationale and Background

The surveys by B. Zelazny in 1975–77 (Zelazny 1980) showed that the incidence of cadang-cadang disease is significantly lower at high altitudes than at sea level. Zelazny considered that this observation could be explained either by a lower rate of spread of the disease at the higher altitudes, or because there is a lower level of disease expression in infected trees at higher altitudes. The second hypothesis was considered to be important because, if it could be verified, it would help explain why a number of coconut palms containing CCCVd-related sequences in countries outside the Philippines (Hanold and Randles 1991) do not show the typical disease syndrome. Therefore, to test the hypothesis that disease expression may be influenced by altitude, the following study was conducted.

Methods and Results

Two plots at approximately sea level and 2 at an altitude of around 200 m were selected in Albay Province. Each consisted of approximately 120 palms with an incidence of cadang-cadang disease (determined by Mr F. Imperial) of between 8% and 16%. Systematic sampling of the symptomless trees in these plots was done by harvesting leaflets from every fifth asymptomatic tree in the rows of the plantation, excluding the diseased trees from the count. The samples were extracted using the method devised for the CCCVd-related RNA survey and assayed by PAGE–electroblot analysis using a cRNA probe (see Chapter 17).

As can be seen from Table 14.1, although the plots had similar levels of cadang-cadang disease, the mean frequency of detection of CCCVd-related nucleic acids in asymptomatic palms was higher in the plots at 200 m altitude and above (41%) than in the plots up to an altitude of 15 m (7.7%). However, one of the high-altitude plots had an incidence that was not significantly different from the low altitude plots; this suggests that factors other than altitude may also influence symptom expression.

These results suggest that some environmental factor such as temperature may affect expression of symptoms of cadang-cadang disease. Further work to study this phenomenon may identify which environmental factors are involved in regulating the expression of typical symptoms of cadang-cadang disease, and may lead to an elucidation of the mechanism of symptom expression.

Table 14.1. Incidence of latent infections with CCCVd at low and high altitude.

Plot	Approximate altitude	Cadang-cadang palms symptomless palms	Incidence of cadang-cadang disease	No. CCCVd positive, symptomless palms*/no. sampled	CCCVd incidence in symptomless palms
A	sea level	16/103	13.4%	3/20	7.7%
B	15 m	20/107	15.7%	0/19	
C	250 m	10/109	8.4%	4/19	41%
D	200 m	11/102	9.7%	12/20	

* Samples showed bands in the viroid region after assay by PAGE–electroblot, hybridisation with cRNA probe, and a high stringency, post-hybridisation wash (see Chapter 17).

Conclusions

This observation has implications for the interpretation of the discovery that CCCVd-related sequences occur in coconut palm and other monocotyledons in countries outside the Philippines (Hanold and Randles 1991), because it suggests that expression of any disease associated with the presence of these viroid-like sequences may be moderated by environmental factors. If this is so, there could be a considerable risk associated with the movement of viroid-positive materials to new environments, since certain environments could permit the expression of severe disease and lead to new epidemics.

15 DESCRIPTION OF THE SURVEY FOR CCCVd-RELATED MOLECULES IN COCONUT PALM AND OTHER TROPICAL MONOCOTYLEDONS

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Introduction

General characteristics of viroids

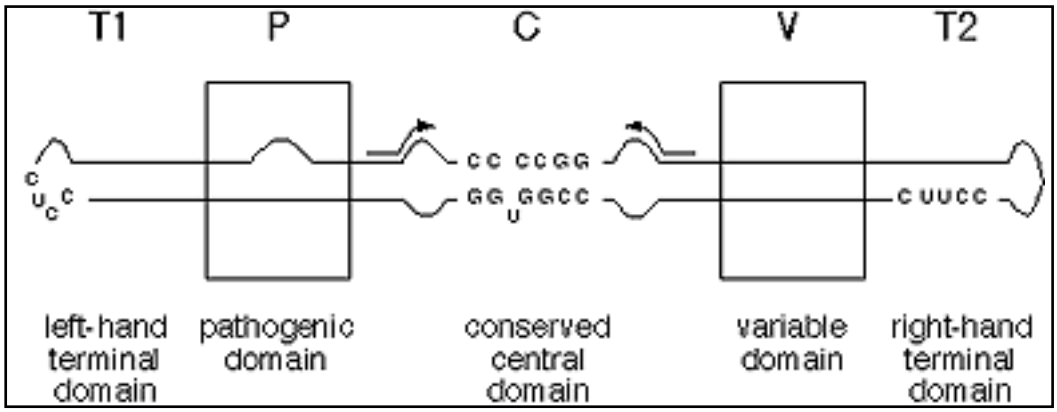
Viroids are the smallest known pathogens and have been found only in plants. Unlike viruses, they do not have a protein coat and consist solely of a small, circular, single-stranded, infective RNA molecule that can both replicate in the host cell and be transmitted independently of any other microorganism. Viroids range in size from 246 to approximately 388 (Symons 1991) nucleotides and have strong, internal nucleotide sequence homologies that lead to substantial base-pairing, which gives them a rod-like shape in their native state. They have a characteristic melting pattern with a transitional intermediate owing to sequences in the conserved region (Riesner 1987a). Owing to their small genome size, their coding capacity is very limited, and they seem to depend entirely on the host's enzymatic systems for their replication. The structure of their RNA and the occurrence of structural transitions seem to enable them to interact with host cell factors (Riesner 1991). Not much is known about the physiology of pathogenicity and the replicative mechanisms of these pathogens, except that they appear to require RNA polymerase II for replication via a rolling circle mechanism, and have no DNA intermediates (Sänger 1987). The concentration of the viroid in the host does not seem to be a measure of pathogenicity for strains of potato spindle tuber viroid (PSTVd) (Diener 1991), indicating that it may be the interference in cellular mechanisms during their reproduction that is harmful to the host, rather than the mature viroid product itself. Most viroids described so far have been found in cultivated plants and are transmitted mechanically by human cultural practices. Some can spread naturally through insects, seed and pollen (Diener 1987a), or by unknown means (CCCVd and coconut tinangaja viroid (CTiVd); Hanold and Randles 1991a).

A model of viroid structure that divides the molecule into a number of domains (Fig. 15.1) has been developed and is now generally accepted (Keese and Symons 1987):

- The central conserved region, C, retains a high level of homology in different viroids belonging to one group, and is thus used to classify viroids.
- The pathogenic region, P, has high levels of homology between viroids with similar host range; mutations in this region can affect infectivity and symptomatology.
- The variable region V.
- The two terminal regions T1 and T2.

When the nucleotide sequence of a new viroid is known, classification can be attempted using the sequence of the central conserved region. On this basis, the viroids have been classified into three major groups: the PSTVd, apple scar skin viroid (ASSVd) and avocado sunblotch viroid (ASBVd) groups (Koltunow and Rezaian 1989, and see Chapter 1).

Figure 15.1. **General model of viroid domains according to Keese and Symons (1987). Arrows indicate an inverted repeat sequence that can form a stem loop (see Fig. 1.5, Chapter 1, for molecular map).**



Diagnosis of viroids, classification into groups and distinction between viroids require comparison of nucleotide sequences (see Chapter 2). The closer the relationship between viroids, the higher is their level of sequence similarity. For example, both CCCVd and chrysanthemum stunt viroid (CSVd) belong to the PSTVd group, and each has approximately 70% similarity in the C region with PSTVd. However, between themselves they share only 64% similarity in C, and 44% similarity overall, and they are thus only distantly related. CCCVd and CTiVd, in contrast, have 77% similarity in C and 64% overall, and are thus closely related. (CTiVd causes a disease in Guam called tinangaja, which resembles cadang cadang.) The CCCVd probe used in the survey described here yielded strong signals with CTiVd, but no reaction with CSVd. We therefore concluded that all viroid-like molecules detected in our survey are more closely related to CCCVd than to CSVd, but at different levels, indicated by the strength of their reaction in the assay. The isolate we have shown to be associated with oil palm 'genetic' orange spotting (GOS), for example, reacts almost as strongly as CTiVd.

Viroids are probably much more widespread than is apparent from the diseases they cause (Hanold and Randles 1991b). Those of current interest have been identified because they are causing serious diseases in crop plants, but it seems probable that many others will not cause obvious symptoms. Such 'latent' viroids may still cause yield reduction or some growth inhibition, and 'latent' hosts may also act as reservoirs for a viroid that can cause disease under changed environmental conditions or in other economically relevant species. Apparent freedom from symptoms cannot be regarded as freedom from viroid pathogens (Hanold and Randles 1991a, (b).

Significance of viroids to the coconut industry

Coconut palm is an essential food, fibre and cash crop in most tropical countries (Persley 1992). World coconut production exceeds 36m t a year from approximately 10m ha. Coconut is mainly a smallholder crop; about 50 million people are involved in its cultivation worldwide. In Asia, another 30 million depend on coconut and its processing as a livelihood. Much of the value of coconut accrues to developing countries.

CCCVd causes a lethal disease of coconut in the Philippines for which no methods of control are available. The ACIAR-sponsored survey during 1988–91 described in this chapter showed the widespread distribution of viroid-like molecules related to CCCVd in the Pacific area in coconut, oil palm and other monocotyledons (Hanold and Randles 1991b). Although no epidemic of a cadang cadang-like disease is known outside the Philippines, this finding has caused major concern in the region. Exchange of coconut germplasm has been stopped because of the possibility that these CCCVd-related molecules may induce disease when transferred to a different environment. Information about potential new viroids, particularly concerning their relationship to CCCVd and to each other, is urgently needed to allow an assessment of their threat to the coconut industry outside the Philippines.

The Survey: Rationale and Limitations

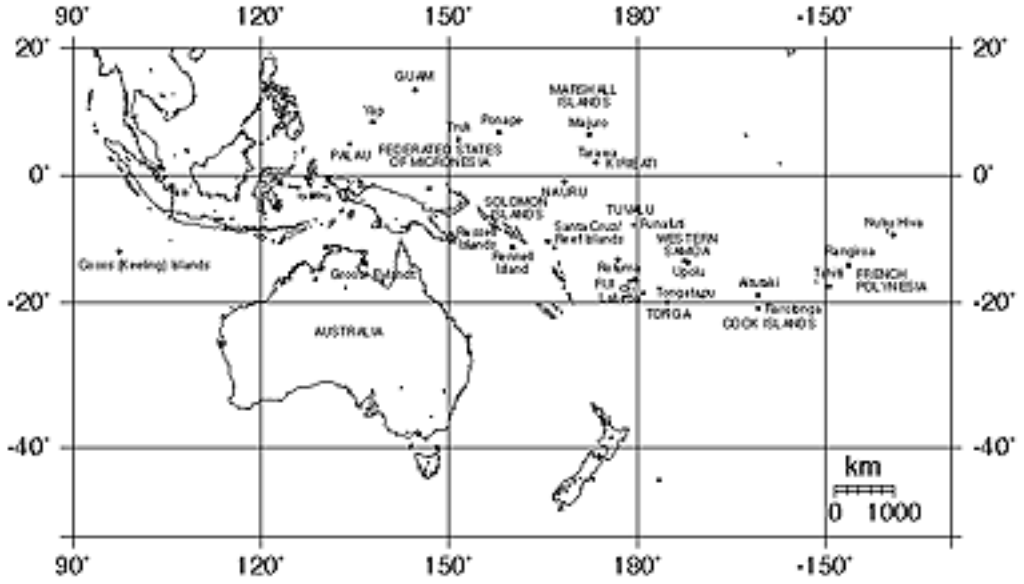
The survey for CCCVd-related sequences was designed to be qualitative, exploratory and extensive, with the aim of covering as wide an area as possible, rather than intensively sampling particular sites. It was to include a range of different ethnic, agricultural, climatic and geographic environments, and to provide the basic information for the planning of more detailed future surveys if required (see Fig. 15.2 for map of collection sites). Samples were screened for both CCCVd-related molecules and CFDV (coconut foliar decay virus)-related viral nucleic acids. Of particular interest were the questions of whether any areas might be apparently free of these pathogen-like nucleic acids, and whether there might be an obvious association of specific environments, host species or disease expression with their distribution.

Only a small number of samples could be included per country, and sample numbers varied, so statistical analyses could not be done. Collection was biased towards abnormal plants that could be sampled. Government authorities in the countries concerned were consulted beforehand and generally supported the work. However, because the release of results was perceived to have a possible deleterious effect on the economy of countries dependent on agriculture for export earnings, confidentiality was required. The results described below will show that confidentiality should not be of future concern, because no area was found to be free of viroid-like nucleic acids. Nevertheless, within different areas, varying percentages of trees sampled were found to be free of viroid-like nucleic acids, and the local strains found in positive palms in different areas seem to differ from each other.

Collection and Storage of Samples

An outline of the sampling procedure is given in Appendix 15.1. Most samples were collected by D. Hanold or J. W. Randles. The rest were delivered or sent by colleagues or local staff (Appendix 15.2, and see Appendix 15.3 for sampling instructions). All material had to be air freighted to Adelaide as fresh leaf, since no preservation method was available at the time of collection. Parts of samples that had turned brown during transport, indicating tissue degradation, were discarded before assay. Overall, samples were obtained from species of 5 families of monocotyledons belonging to 4 superorders. A total of 2133 samples were collected from 53 sites in 29 countries (Appendix 15.4). Sites were approximately 0.25–1 km^2 in area, and were either located on separate islands or separated by a distance of at least 10 km (often more on large islands). A summary of the entire sample collection is given in Table 15.1.

Figure 15.2. Map of survey collection sites.



Leaflets of palms were harvested from fronds about 10 positions below the unopened spear leaf; for herbaceous plants, young, fully expanded leaves were chosen. Midribs were discarded, and approximately 50 g of tissue was sealed in polythene bags for transport and stored at –20°C on arrival. At the end of the project, when all analyses had been completed, we devised a tissue preservation method, which has been used to preserve aliquots of all positive and a few selected negative samples for future reference. This allowed us to discard surplus frozen samples.

Coconut palms were sampled in 27 of the 29 countries. An effort was made to collect leaves from older trees (although height is a serious obstacle), since CCCVd incidence in the Philippines is much lower among young palms. In addition, because CCCVd is a slow pathogen, trees with slowly developing symptoms such as stunting or tapering of the trunk were selected if possible. Rapidly developing symptoms (such as leaf necrosis) are more likely to be caused by faster developing pathogens (e.g. fungi), or by pests, or by restrictive growth conditions (e.g. water stress, nutrient-deficient coral rock or cyclone damage). Ideally, 40–50 coconut palms would be sampled per country from several sites, both cultivated and in a state of native vegetation, but this was not always achievable. Detailed notes were made for each site and tree sampled (Appendix 15.5), and a photograph was taken in most cases. Individual trees were marked for identification whenever it could be arranged, and local staff were instructed concerning possible resampling.

Other monocotyledons were also sampled in each area if possible:

- Other palm species, particularly those known to be alternative hosts for CCCVd. Abnormal plants were sampled preferentially, and oil palms with orange leaf spotting (GOS) were selected.
- Pandanus, owing to its wide distribution near the coast throughout the Pacific, and the fact that it frequently grows in association with coconut palms. Species were not identified, but both tree-like and large-leaved creeping forms were sampled. None of the *Pandanus* spp. sampled showed disease.

- Herbaceous monocots, including gingers (Zingiberaceae), grasses (Poaceae), Commelinaceae, and arrowroot (Marantaceae), principally because in the Philippines they have been shown to contain CCCVd-related molecules. Some are also susceptible to mechanical inoculation but do not develop any symptoms. They are frequently associated with coconut palms, and could possibly act as alternative hosts for viroids. None of these herbaceous species showed any obvious abnormalities.

In July 1992, preliminary results for coconut palms only were sent out to all participants (see Appendix 15.6) with code numbers instead of country names to ensure confidentiality while appropriate action was being considered. Final analysis of all data was completed 6 months later. Because of small sample numbers in most instances, statistical analyses were attempted but could not be interpreted. Nevertheless, trends and indications have become apparent that now need to be confirmed by large-scale experiments designed for the purpose. Overall, the survey proved most fruitful in exploring the viroid situation, and this will greatly benefit all coconut-producing countries, if the work can be continued.

Explanations and Conclusions

- The table shows the number of samples that contain nucleic acid molecules closely related to CCCVd by size and molecular structure.
  Samples from different areas may contain different distinct types of these molecules, all related to CCCVd and to each other, but not identical. Further studies are needed to classify and characterise the various members of the family and to assess what risk they pose to the countries.
- In most countries, samples of species other than coconut were also collected and analysed, including oil and other palms, *Pandanus*, gingers, grasses and arrowroot. Some of these also contained CCCVd-related molecules that need to be compared with the coconut isolates to determine whether the other species could serve as reservoirs. For oil palm, severe orange leaf spotting, reduction of yield and stunting (previously called ‘genetic’ orange spotting syndrome) was shown to be associated with a viroid related to CCCVd.

Analysis of relationships between viroid detection and origin, cultivar and age of palms is proceeding.

Table 15.1. **Summary of number of samples in survey for CCCVd-related molecules.**

Country* & site		Coconut	Oil palm	Other palms	Pandanaceae	Zingiberaceae	Marantaceae	Commelinflorae	Other monocots	Total no. samples
Australia (AUS)	1	1		32	4				4	
	2	36	1	4	7	6		3	7	
	3			3					3	
	4	39			1	3	3	5	3	
	5	5		2	4					
	6	10			4			3	7	
	total	91	1	41	20	9	3	11	24	200
Cook Is (COK)	1	15			1	2	1		2	
	2	16			3			1	3	
	total	31			4	2	1	1	5	44
Costa Rica (CRI)		6							6	6
Fiji (FJI)	1	19			5	7			2	
	2	32	1	5	6	9	2	2	3	
	3	24			8	3			3	
	4	31		1	5	5	1		3	
	total	106	1	6	24	24	3	2	11	177
French Polynesia (PYF)	1	26		1	2	8		4	7	

Table 15.1. (cont'd) (cont'd) Summary of number of samples in survey for CCCVd-related molecules.

Country* & site		Coconut	Oil palm	Other palms	Pandanaceae	Zingiberaceae	Marantaceae	Commelinflorae	Other monocots	Total no. samples
	2	21			6		1		2	
	3	17	1	4	6	3		3	3	
	total	64	1	5	14	11	1	7	12	115
Guam (GUM)		38		6	1			4	1	50
India (IND)			8							8
Indonesia (IDN)	1	23	2		1		1		1	
	2	6	40		1	1	2		1	
	3	7	1							
	total	36	43		2	1	3		2	87
Kiribati (KIR)		43			10					53
Malaysia (MYS)		10	16		1	2	1		4	34
Marshall Is (MHL)		28			9			1		38
Mozambique (MOZ)		5								5
Nauru (NRU)		6		1	1				1	9
New Caledonia (NCL)	1	16		1						
	2	13								
total		29		1						30
New Zealand (NZL)				1						1

Table 15.1. (cont'd) (cont'd) Summary of number of samples in survey for CCCVd-related molecules.

Country* & site		Coconut	Oil palm	Other palms	Pandanaceae	Zingiberaceae	Marantaceae	Commelinflorae	Other monocots	Total no. samples
Papua New Guinea (PNG)	1	2								
	2	30			2	15	2	3		
	total	32	56		2	15	2	3		110
Palau (PLW)†		2		3						5
Philippines (PHL)	1	80								
	2	12				2				
	3	63	24	2	1		2	2	1	
	total	155	24	2	1	2	2	2	1	189
Ponape (PON)†		9		1	2				2	14
Solomon Is (SLB)	1	24	374	4		2		7	8	
	2	87			1	16		1		
	3	12			1					
	4	24		4						
	total	147	374	8	2	18		8	8	565
Sri Lanka (LKA)		10								10
Tanzania (TZA)		15								15
Thailand (THA)	1	45	4							
	2	4	8		1	2				
	3		4							
	total	49	16		1	2				
Tonga (TON)		25			6	4		1	6	42
Truk (TRU)†		2								2
Tuvalu (TUV)		40			10					50
Vanuatu(VUT)	1	90	3	13	13	5	1	4	13	

Table 15.1. (cont'd) (cont'd) Summary of number of samples in survey for CCCVd-related molecules.

Country* & site		Coconut	Oil palm	Other palms	Pandanaceae	Zingiberaceae	Marantaceae	Commelinflorae	Other monocots	Total no. samples
	2	4			1					
	3	4			6					
	total	98	3	13	20	5	1	4	13	13
Western Samoa (WSM)		26			7	10		2	1	46
Yap (YAP)†	2	1								3
Total:										
29	53	1105	543	89	137	10 5	17	46	91	2133

* Country abbreviations follow the ISO 3166 standard (except PON, TRU and YAP).
†Federated States of Micronesia (FSM).

APPENDIX 15.1—SAMPLING PROCEDURE FOR VIROID AND VIRUS SURVEY

Definitions

- *Area* refers to a country, province, group of islands or island and is decided when planning the survey.
- A *site* usually measures between 200 × 200 m and 500 × 500 m. Different sites are separated by at least 10 km or are on different islands. At least 2 sites should be sampled for each area.

Samples per site

- 5 coconut palms
- Up to 3 of any other palm species if present (e.g. oil palm, sago, buri); minimum 1 sample per species
- 3 samples of other monocotyledons
- 1–2 *Pandanus* if present.

Selection of plants

Coconuts

Look for the following symptoms (code):
- Stunting (st)
- Chlorosis of the crown (cc)
- Reduced nut production or no nuts rnp or (nn)
- Translucent, yellow, **non-necrotic** leaf spots (ls)
- Necrosis of inflorescences (in)
- Abnormal nuts, e.g. spindle shape, scarifications, rounding (specify) (an)

If any of these symptoms are present on 1 or more palms, collect samples (up to 3 per site). Also sample a minimum of 2 palms of normal appearance.

Other palms

Look for:

- stunting
- **non-necrotic** leaf spots (orange–yellow)
- reduced bearing.

If symptomatic palms are present, sample up to 2 per species and at least 1 healthy-looking palm (if present).

Weeds

Order of preference of species:

1. Gingers or related species (Zingiberaceae), e.g. wild ginger, *Alpinia*, cardamom
2. Arrowroot (*Maranta*)
3. *Commelina* sp.
4. Other large-leafed monocots (e.g. *Canna*)
5. Grasses.

Minimum of 1 sample per species in 1–4; sample grasses (no. 5) only if no other species are present. (Take 3 plants, preferably of different grass species.) Examples:

- For a site with 1 ginger species and grasses, sample 3 gingers only; total of 3 samples.
- For a site with 2 ginger species, arrowroot, *Commelina* sp. and grasses, 1 sample of each ginger sp. and 1 sample of the arrowroot; total of 3 samples.

Pandanus

- If abnormal plants present: 1 abnormal, 1 healthy.
- If many healthy plants: 2 samples.
- If few (up to 5 total per site): 1 sample.

Sampling

Mark sampled trees with painted numbers whenever possible.

If more than 1 plant per species is sampled, individual plants should be spaced out through the site.

Make **exact** notes for each sample in the survey form (Appendix 15.3):

1. Genus andspecies (if known); local name; photograph if possible.
2. Details of age, variety (e.g. MRD), origin of seed material.
3. Properties such as symptoms, general appearance, vigour, any peculiarities (e.g. injuries, bent etc.); also note if asymptomatic.
4. Describe location: name and owner of property; surroundings (e.g. ‘near village’, ‘on creek bank’); state of upkeep (e.g. high grass).
5. General health of all plants (insect attacks, nutritional problems).
6. Soil properties (e.g. rocky, loam, swampy) and situation (e.g. hillside, north-facing).
7. For palms: usual method of fruit harvest (e.g. climber, long knife, natural fall).
8. Any other observations.

Sample 50 g of leaf without midribs and stems:

- Palms and *Pandanus*: mid crown (around frond no. 10) or with good symptoms if present; leaflets from mid frond.
- Weeds: young, fully expanded leaves.

Keep all harvested material cool, in shade, and seal in plastic bags within 30 min. of harvest. Keep all packaged samples out of direct sun and store in air-conditioned room before shipping. Specifically:

1. Remove dirt, insects and necrotic tissue and wipe off all surface moisture.
2. Seal in plastic (air-tight to prevent drying out).
3. Keep samples cool and ship within 2 days! Insulate outside of parcel (e.g. newspaper layer). Attach copy of quarantine permit, mark 'perishable' on waybill and parcel, and fax waybill number to indexing laboratory at the Waite Agricultural Research Institute, University of Adelaide.

APPENDIX 15.2—COLLECTIONS AND CONTACTS

Australia

Collections

Cocos (Keeling) Islands	B. Leach	10.90
Cocos (Keeling) Islands	P. Stevenson	5.2.91
Groote Eylandt	O. Foale	13–15.5.91
Darwin	K. Gibb	28.3.91
Darwin	T. Lim	3.91
Darwin	B. Conde	22.4.92
Jabiru, Alice Springs	J. Randles	7.92
Palm Valley	P. Turner	13.4.91
Far North Queensland	D. Hanold, J.W. Randles	2.91
Adelaide Botanic Gardens	D. Hanold	12.89, 1.3.91

Contacts

P. Stevenson, National Parks and Wildlife Service, Cocos (Keeling) Islands
Department of Primary Industry and Fisheries, Berrimah, NT
South Johnstone and Kamerunga Research Stations, Queensland Department of Primary Industry

Cook Islands

Collections

Rarotonga, Aitutaki	J. Randles	5–7.7.90

Contacts

P. Joseph, Ministry of Agriculture, Rarotonga
F. Charlie, Ministry of Agriculture, Aitutaki

Costa Rica

Collections

Atlantic Zone	R. Illingworth	1.7.91

Contacts

R. Illingworth, SACRAC, San Jose

Fiji

Collections

Viti Levu	D. Hanold, J. Randles	11–27.6.90
Viti Levu	D. Hanold	6–13.7.90
Vanua Levu, Taveuni	D. Hanold	23–28.7.90
Lakeba (Lau Islands)	D. Hanold	7–10.8.90
Rotuma Island	D. Hanold	14–21.7.90

Contacts

J. Kumar and F. Vilsoni, Koronivia Research Station, Nausori
R. Macfarlane and Dr G. Jackson, South Pacific Commission

Federated States of Micronesia

Palau

Collections

	G. Jackson	6.90

Contacts

via Plant Protection Service, South Pacific Commission, Suva, Fiji

Ponape

Collections

	G. Jackson	14–16.6.90

Contacts

via Plant Protection Service, South Pacific Commission, Suva, Fiji

Truk

Collections

	G. Jackson	6.90

Contacts

via Plant Protection Service, South Pacific Commission, Suva, Fiji

Yap

Collections

	G. Jackson	6.90

Contacts

via Plant Protection Service, South Pacific Commission, Suva, Fiji

French Polynesia

Collections

Nuku Hiva (Marquesas Islands)	D. Hanold, J. Randles	29.6.90
Rangiroa	J. Randles	2.7.90
Tahiti	D. Hanold	2–3.7.90

Contacts

Dr C. Garnier, Service Economie Rurale, Papéété

S. Amiot, Service Economie Rurale, Nuku Hiva

M. Pomier, Service Economie Rurale/IRHO

Guam

Collections

	G. Jackson	31.5.90
	G. Wall	17.6.91

Contacts

Dr G. Wall, University of Guam

India

Collections

Kerala	K. Babu	18.12.90, 2.2.91

Contacts

K. Babu, Central Plantation Crops Research Institute, Kerala

Indonesia

Collections

Central Kalimantan	D. Hanold	15.5.89
West Java, North Sumatra	J. Randles	7–15.9.90

Contacts

Ir. Sumarna, Dinas Perkebunan, Bandung
Dr D. Sitepu, Plant Disease Subdivision, Bogor
H. Runtungan, Agricultural Agency for Research and Development, Sukubumi
J. Mogea, Herbarium Bogoriense, Bogor
Dr K. Pamin, RISPA, Medan
Dr M. Soehardjan, Director of Estate Crop Protection
Prof. G. Varghese, P.T. Hasfarm Dian Konsultan, Jakarta

Kiribati

Collections

Tarawa	D. Hanold	18–24.8.90

Contacts

S. Edwards and Dr G. Sandhu, Ministry of Natural Resources Development, Tarawa
H. Bowman, Australian High Commission

Malaysia

Collections

Selangor	J. Randles	19–28.3.90

Contacts

Mohamed Rof bin Mohd Nor, MARDI Research Station

Chung Gait Fee, Sime Darby Plantations

Roger Naidu, PORIM

Golden Hope Research Station, Banting

Marshall Islands

Collections

Majuro	D. Hanold	13–17.8.90

Contacts

J. Joseph and D. Garel, Department of Resources and Development, Majuro

Mozambique

Collections

Zambesia Province	M. Foale	27.3.92

Contacts

Madal Plantation, Quelimane, Zambesia Province

Nauru

Collections

	S. Foale	7.2.91

Contacts

None

New Caledonia

Collections

Nouméa, Ouvéa	J. Randles	17–21.6.90

Contacts

R. Amice, Service Vétérinaire du Contrôle de la Qualité des Produits Agro-Alimentaires
D. Burrus, Service de l'Agriculture des Forêts et des Pêches, Province des Iles Loyauté

New Zealand

Collections

North Island	D. Hanold, J. Randles	2.90

Contacts

Gordon Grandison, DSIR, Auckland

PNG

Collections

New Britain	D. Hanold	27.11–6.12.88
New Britain	D. Hanold, J. Randles	12.89
Port Moresby area	T. Ovasuru	14.2.91

Contacts

Cocoa and Coconut Research Institute, Keravat

Philippines

Collections

Socorro (Mindoro Island)	D. Hanold	20–21.3.90
UPLB Los Baños	D. Hanold	16.9.88
Davao, Zamboanga, Basilan Id (Mindanao)	D. Hanold	28.2–3.3.90

Contacts

Philippine Coconut Authority, Albay, Davao and Zamboanga Research Centres, and head office, Manila

Solomon Islands

Collections

Guadalcanal	J. Randles	6.87, 12.89, 1.91
Guadalcanal	D. Hanold	22.5–15.6.89
Russell Islands, Guadalcanal, Santa Cruz	J. Randles	10.87
Russell Islands, Guadalcanal	D. Hanold	12–27.11.88
Reef Islands, Santa Cruz, Guadalcanal	G. Jackson	26.3.88
Rennell Island	J. Saelea	6.5.91

Contacts

R. Liloquila and J. Saelea, Ministry of Agriculture and Land
I. Rankine, Solomon Islands Plantations Ltd
D. Friend, Unilever, Yandina

Sri Lanka

Collections

Colombo area	K. Jayasena	6.11.90

Contacts

R. Mahindapala, Coconut Research Institute, Lunuwila

Tanzania

Collections

Zanzibar	M. Foale, M. Juma	11.4.92

Contacts

Mwatima A. Juma and Dr H. Harries, National Coconut Development Programme

Thailand

Collections

Gulf of Thailand, Phuket, Haadyai	J. Randles	6–19.3.90
Chumphon	P. Wongwathanarat	4.90

Contacts

Dr Duangchai Choopanya, Department of Agriculture, Bangkok

Tonga

Collections

Tongatapu	D. Hanold	30.7–1.8.90

Contacts

S. Pone and K. Englberger, Ministry of Agriculture, Forests and Fisheries, Nuku‘alofa

Tuvalu

Collections

Funafuti	D. Hanold	25.8–4.9.98

Contacts

T. Teii, Ministry of Commerce and Natural Resources, Funafuti

Vanuatu

Collections

Santo	D. Hanold	25.10–11.11.88
Santo, Efate, Tanna	J. Randles	12.89
Santo	J. Randles	9–17.6.90
Santo	J. Randles	29.2–14.3.92

Contacts

J. P. Morin and C. Calvez, Saraoutou Experimental Station, IRHO, Espiritu Santo

Western Samoa

Collections

Upolu	D. Hanold	2–4.8.90

Contacts

S. Aveau, S. Matalavea and S. Efu, Department of Agriculture, Forests and Fisheries, Apia

APPENDIX 15.3—SURVEY FORM

Samples for viroid and virus survey

Area:

Date:

Site:

Sampled by (name & address):

Sample no.	1* species	2* details	3* properties	4* location	5* general health	6* soil	7* harvest	8* remarks

–See Appendix 15.1, ‘Sampling’

APPENDIX 15.4—LOCATIONS

Country	Code*	Site
Australia	AUS	1 tropical Northern Territory 2 far north Queensland 3 Alice Springs 4 Adelaide/unknown 5 Groote Eylandt 6 Cocos (Keeling) Islands
Cook Islands	COK	1 Rarotonga 2 Aitutaki
Costa Rica	CRI	Atlantic zone
Fiji	FJI	1 Vanua Levu, Taveuni 2 Viti Levu 3 Lakeba 4 Rotuma
Federated States of Micronesia	FSM	
Palau	PLW	
Ponape	PON	
Truk	TRU	
Yap	YAP	
French Polynesia	PYF	1 Tahiti 2 Rangiroa 3 Nukuhiva
Guam	GUM	
India	IND	Kerala
Indonesia	IDN	1 West Java 2 North Sumatra 3 Central Kalimantan
Kiribati	KIR	Tarawa
Malaysia	MYS	Selangor
Marshall Islands	MHL	Majuro
Mozambique	MOZ	Zambesia Province
Nauru	NRU	
New Caledonia	NCL	1 Nouméa 2 Ouvéa
New Zealand	NZL	North Island
Papua New Guinea	PNG	1 Port Moresby 2 New Britain
Philippines	PHL	1 Luzon 2 Mindoro Island 3 Mindanao

Solomon Islands	SLB	1 Guadalcanal 2 Russell Islands 3 Rennell Island 4 Santa Cruz/Reef Islands
Sri Lanka	LKA	Colombo area
Tanzania	TZA	Zanzibar
Thailand	THA	1 Gulf of Thailand 2 Phuket and surroundings 3 Haadyai
Tonga	TON	Tongatapu
Tuvalu	TUV	Funafuti
Vanuatu	VUT	1 Espiritu Santo 2 Efate 3 Tanna
Western Samoa	WSM	Upolu

*Country abbreviations follow the ISO 3166 standard (except PON, TRU and YAP).

APPENDIX 15.5—CODING SYSTEM USED TO DESCRIBE TREE CHARACTERISTICS IN THE SURVEY

Code	Characteristic	Description
Leaf symptoms:		
41	Pin-pricks	0—no; 1—few; 2—many
42	Non-necrotic leaf spots	0—no; 1—few; 2—many
43	Chlorosis	0—no; 1—mild; 2—severe
44	Necrosis of leaflets	0—no; 1—yes
45	Other	0—no; 1—other symptoms; 2—no information
46	Defined syndrome	0—no; 1—yes
Crown symptoms:		
47	Frond necrosis	0—no; 1—on few; 2—on many
48	Frond breakage	0—no; 1—yes
49	Short fronds	0—no; 1—yes
50	Reduced	0—no; 1—yes
51	Ruffled	0—no; 1—yes
52	Other	0—no; 1—other symptoms; 2—no information
Stem symptoms:		
53	Stunting	0—no; 1—yes
54	Tapering	0—no; 1—yes
55	Constriction	0—no; 1—yes
56	Other	0—no; 1—other symptoms; 2—no information
Nut characteristics:		
57	Reduced number	0—no; 1—few nuts; 2—none
58	Small size	0—normal size; 1—smaller than normal
59	Abnormal shape	0—no; 1—rounded; 2—elongated
60	Scarifications	0—no; 1—yes
61	Necrotic inflorescences	0—no; 1—yes
62	Other	0—no; 1—other characteristics; 2—no information
Age of tree:		
63	Age	0—unknown; 1—less than 5 y; 2—adult tree
Variety:		
64	Tall	0—no; 1—unknown variety; 2—local; 3—RT; 4—other; 5—no information
65	Dwarf	0—no; 1—unknown variety; 2—local; 3—MRD; 4—MYD; 5—other; 6—no information 0—no; 1—yes ; 2—no
66	Hybrid	information

APPENDIX 15.6—FORM LETTER AND CODED RESULTS SENT TO PARTICIPATING COUNTRIES

THE UNIVERSITY OF ADELAIDE
WAITE AGRICULTURAL RESEARCH INSTITUTE
DEPARTMENT OF CROP PROTECTION
(Entomology, Plant Pathology & Weed Science)

Head of Department:
Professor Otto Schmidt

Telephone: +61-8-372 2269/70
Facsimile: +61-8-379 4095

Wednesday, July 15, 1992

RE: ACIAR Survey of Coconut Viroids Related to Cadang-Cadang

Please find enclosed a table of viroid incidences for the countries surveyed. Countries are listed at random.

Your country .....

is represented by the number .....

Sites: .....

.....

.....

.....

Complete information identifying all the countries has been sent, in confidence, to R Macfarlane, SPC, only.

We will publish a monograph at the end of this year giving detailed results, conclusions and recommendations of which you will receive a copy. In the meantime, if you have any queries, please contact R Macfarlane.

Thank you very much for your co-operation and for participating in our survey. It has been most fruitful and will benefit all coconut growers.

Dr D Hanold **Dr JW Randles**

Coconut samples containing nucleic acids related to CCCVd according to countries and sites; tested by hybridisation assay at high stringency.

Country	Site	No. samples	Positive number	% of total
1	1	12	2	17
	2	63	36	57
2		38	16	42
3	1	45	32	71
	2	4	3	75
4		10	9	90
5	1	24	8	33
	2	6	5	83
	3	7	5	71
6		10	4	40
7	1	2	2	100
	2	30	13	43
8	1	24	18	75
	2	87	18	21
	3	12	6	50
	4	19	3	16
9	1	43	9	21
	2	4	1	25
	3	4	0	0
10	12	16	9	56
		13	9	69
11	1	55	31	56
	2	5	2	40
12	1	32	25	78
	2	19	16	84
	3	23	13	57
	4	31	18	58
13		25	13	52
14		26	11	42
15	1	15	11	73
	2	16	10	63
16	1	26	11	42
	2	21	6	29
	3	17	9	53
17		40	9	23
18		6	4	67
19		43	16	37
20		28	7	25
21		9	8	89
22		2	1	50
23		2	0	0
24		2	2	100
25		10	9	90
26		6	4	67
Total	44	932	444	48

16 METHODS USED FOR ASSAY OF THE SAMPLES COLLECTED DURING THE SURVEY

D. Hanold* and J. W. Randles*

* Department of Crop Protection, Waite Campus, University of Adelaide, Glen Osmond, South Australia 5064, Australia, Fax: +61 8 8379 4095

Purification, Electrophoretic Separation and Detection of Viroid-Like Nucleic Acids in the Survey Samples

Chapter 2 describes sampling and storage methods, and the general instructions given there for work with RNA were applied here. The protocol successfully used for the extraction of CCCVd, CTiVd and related viroids from palms and herbaceous monocotyledons is given in Chapter 2, Table 2.1. Extracts were stored at –20°C until the time of analysis.

Nucleic acid samples from the survey were fractionated by electrophoresis in 5% or 20% polyacrylamide gels (PAGE) buffered in TBE. The CCCVd standard was prepared from infected palms in the Philippines and mixed to provide similar amounts of each of the 246-, 247-, 296- and 297-nucleotide forms and their respective dimers. Non-denaturing/denaturing, 2-dimensional 5% PAGE was done according to Schumacher et al. (1983). Temperature gradient electrophoresis was carried out as described in Chapter 2.

An attempt was made to use a simplified agarose gel electrophoresis method instead of PAGE for screening the survey samples, but it was unsuccessful, because the procedure was neither sensitive nor accurate enough for reliable detection of CCCVd-related nucleic acids.

With the survey material, the only suitable detection method was Northern blotting of gels and subsequent molecular hybridisation assay using a CCCVd probe. Blots were prepared as described in Chapter 2, and the hybridisation protocol is given in Chapter 2, Table 2.3.

A complementary RNA (cRNA) probe labelled with ^{32}P was prepared from a pSP64 plasmid containing a monomeric insert of the 246-nucleotide form of CCCVd at the BamHI site. Transcription was done with a kit from Bresatec, Adelaide, and the RNA was isolated on a 5% polyacrylamide gel containing TBE and eluted for 16 h at 37°C in 500 mM ammonium acetate, 1 mM EDTA, 1 g/L SDS (Maxam and Gilbert 1980). The probe was precipitated with ethanol and resuspended in 10 mM Tris·HCl (pH 8.0), 0.1 mM EDTA, 1 g/L SDS.

The blots were hybridised, washed at low stringency, and autoradiographed for 2–3 days, then rewashed at high stringency, and autoradiographed again for several weeks.

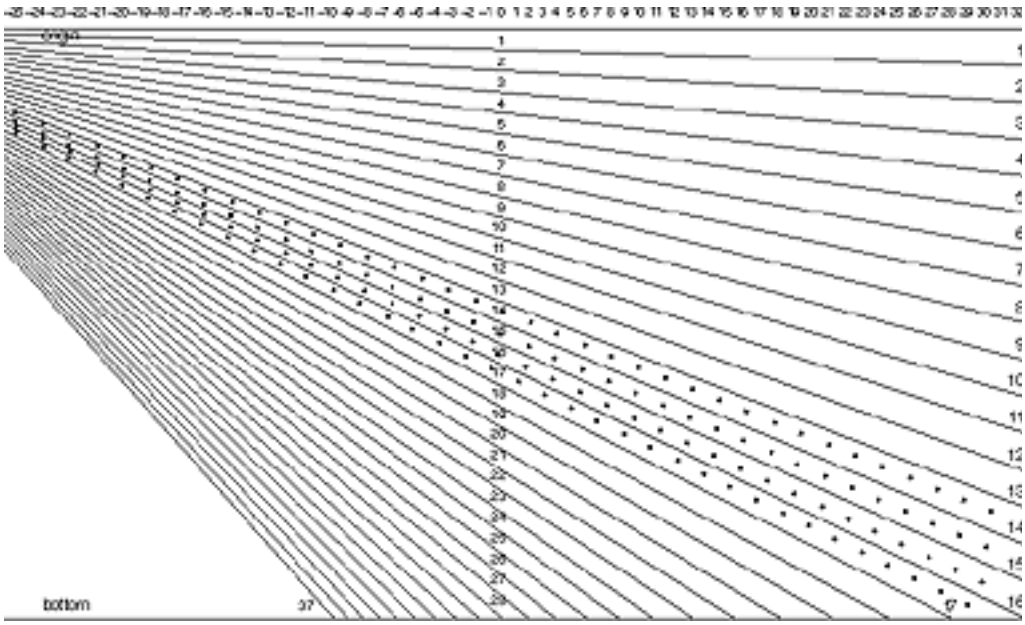
Interpretation of Banding Patterns

The 2 main aspects we took into consideration when trying to interpret the banding patterns were the strength of binding of CCCVd probe and the electrophoretic mobility relative to purified CCCVd markers.

At high stringency, the probe used showed minimal loss of signal strength (compared with low stringency) from the CCCVd markers, approximately 80% loss from CTiVd markers, and no

signal for CSVd. We therefore considered the probe to have high specificity for CCCVd and its close relatives at high stringency.

Figure 16.1. **Template used to compensate for variations in running conditions when comparing band mobilities in different gels to standard CCCVd markers (see text for explanation). CCCVd monomers in zones 14 (296), 15 (297), 16 (246) and 17 (247); CCCVd dimers in zones 7 and 8; top line represents origin of gel.**



We regarded the initial signal intensity from each band to be due to a combination of quantity of viroid-like nucleic acid and its capacity to hybridise to the probe. We used the subsequent rate of probe loss from each band relative to the CCCVd markers when comparing low and high stringency washes of the blots to estimate the degree of nucleotide homology and hence relatedness of the sequence of the viroid-like nucleic acid in the band to CCCVd. A low level of sequence homology, for example, would result in incomplete, and therefore weak, hybridisation with the probe, leading to rapid loss of signal intensity with increasingly stringent binding conditions.

The relative electrophoretic mobility provides an indication of similarity of size or molecular structure of nucleic acids. Figure 16.1 shows the template used for comparing mobilities of bands in sample tracks with the standard CCCVd markers. Gels cannot be compared directly since there is some unavoidable variation in conditions between runs and thus between band mobility, due to environmental influences (e.g. temperature fluctuations, polyacrylamide pore size) and human factors. Therefore every gel incorporated an internal standard track as a point of reference. The translucent template was adjusted over the autoradiograph so that the central 4 zones (marked with dots) coincided with the 4 CCCVd bands of the respective marker track, and relative positions of bands in the sample tracks were read according to the numbered subdivisions.

Both ability to retain probe at high stringency (as a measurement of the degree of relatedness of nucleotide sequences) and the position of a band in the gel (as a measurement of the similarity

of molecular size and structure) were taken into consideration when judging the degree of relatedness between a viroid-like nucleic acid from a sample and the known CCCVd. For example, a band in the CCCVd region of the gel that showed 90% of the CCCVd marker's ability to retain its low-stringency signal intensity after high-stringency washing would be considered closely related to CCCVd, whereas a band above or below the CCCVd region that lost most of its relative signal strength during rewashing at high stringency would be considered only distantly related to CCCVd. In both cases, the initial signal strength after a low-stringency wash gives an estimate of the amount present in the band relative to that bound by all the bands in the gel, but it is not necessarily a direct measure of the quantity in the plant, since different tissues vary in content and may be extracted with different levels of efficiency.

17 RESULTS OF THE SURVEY FOR COCONUT PALM

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Introduction

Evaluation of the 1988–91 survey has shown that a range of molecules both closely and distantly related to CCCVd are detectable in all countries and in about 50% of all samples by gel blot hybridisation assay using a full-length probe for CCCVd. Incidence appears to vary from country to country, and between areas within countries. Of particular concern is a high incidence in the Thailand–Malaysian–western Indonesian region, from which dwarf parents have been obtained for breeding purposes by many countries. A hypothesis that may explain the high incidence of CCCVd-like RNA sequences in all the coconut-growing countries surveyed is that the viroids may be transferred in germplasm to new plantations and subsequently locally distributed by nuts or transmitted by pollen to other trees.

Analysis of our survey samples has revealed complex patterns of molecular species binding the CCCVd probe, and the interpretation of these data is impossible without knowing their nucleotide sequences. It has been found in other host species, such as citrus and grapevine, that multiple viroid infections occur and that pathogen interactions in these situations are complex. The components of multiple infections can be independent, can help or inhibit each other's replication, or can induce different symptoms alone or in co-infection. In the case of single infections, isolates prepared from one host plant often contain several sequence variants of the viroid (Symons 1991) that can be cloned separately. On the other hand, CCCVd exists in well-defined, progressively different molecular forms depending on the stage of disease. It will be necessary to distinguish between these possibilities for any new viroids when assessing the epidemiological risks and deciding what recommendations to suggest for the exchange of coconut germplasm.

Presence of Molecules Related to CCCVd

A total of 1024 coconut samples from 48 sites in 27 countries were assayed by the method described in Chapter 16, and the results are shown in Table 17.1. Figure 17.1 shows an example of an autoradiograph after low- and high-stringency washes. Sample tracks were scored for presence of bands both overall and in the viroid region; that is, the region of the gel that was defined by the highest and lowest of the CCCVd monomer marker bands; it has to be stressed that bands were listed as being merely in the same region as the viroid bands, but not necessarily in exactly the same place; thus they cannot automatically be considered to represent molecular species identical to CCCVd forms or to each other.

Table 17.1 Survey results for coconut samples (PAGE–hybridisation assay at high stringency using a CCCVd probe).

Locations included in the survey			Percentage with CCCVd-related nucleic acids			
Country	**Site**	**No. samples**	**Sample overall**		**Viroid region**	
			Low stringency	**High stringency**	**Low stringency**	**High stringency**
Australia	2 Far north Queensland	56	82	75	75	55
	5 Groote Eylandt	5	80	60	80	40
	6 Cocos (Keeling) Islands	10	80	80	50	40
	total	71	82	75	72	54
Cook Islands	1 Rarotonga	15	100	93	73	73
	2 Aitutaki	16	94	94	81	63
	total	31	97	96	77	68
Costa Rica	Atlantic Zone	6	100	83	83	67
Federated States of Micronesia—see Palau, Ponape, Truk, Yap						
Fiji	1 Vanua Levu and Taveuni	19	95	89	79	84
	2 Viti Levu	32	94	84	81	78
	3 Lakeba	23	100	91	91	57
	4 Rotuma	31	87	84	71	58
	total	105	93	91	80	67
French Polynesia	1 Tahiti	26	100	92	46	42
	2 Rangiroa	21	71	67	43	29
	3 Nukuhiva	17	94	88	76	53
	total	64	89	83	53	45
Guam		38	100	84	74	42
India	Kerala	0	0	0	0	0
Indonesia	1 West Java	24	100	75	46	33
	2 North Sumatra	6	100	83	83	83
	3 Central Kalimantan	7	100	100	100	71
	total	37	100	81	62	49
Kiribati	Tarawa	43	84	81	40	37
Malaysia	Selangor	10	100	90	100	90
Marshall Islands	Majuro	28	100	89	57	25
Mozambique	Zambesia Province	5	100	40	40	40
Nauru		6	100	100	100	67
New Caledonia	1 Nouméa	16	56	56	56	56
	2 Ouvéa	13	69	69	69	69
	total	29	62	62	62	62
New Zealand	North Island	0	0	0	0	0
Papua New Guinea (a = only 13 assayed)	1 Port Moresby	2	100	100	100	100

Table 17.1 (cont'd) Survey results for coconut samples (PAGE–hybridisation assay at high stringency using a CCCVd probe).

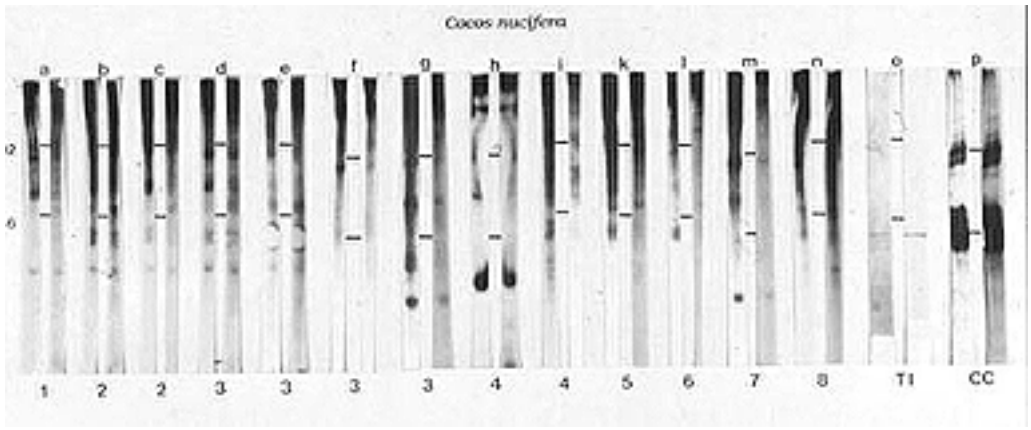
Locations included in the survey			Percentage with CCCVd-related nucleic acids			
Country	Site	No. samples	Sample overall		Viroid region	
			Low stringency	High stringency	Low stringency	High stringency
	2 New Britain	30	83	77[a]	77	43[a]
	total	32	84	92[a]	78	85[a]
Palau		2	100	100	100	100
Philippines	1 Luzon	78	99	95	69	33
	2 Mindoro Island	12	67	33	50	17
	total	153	95	75	78	42
Solomon Islands (a = only 76 assayed) (b = only 22 assayed) (c = only 33 assayed) (d = only 9 assayed)	1 Guadalcanal	25	96	95[b]	96	72[b]
	2 Russell Islands	68	90	55[c]	68	26[c]
	3 Rennell Island	12	92	67	83	50
	4 Santa Cruz and Reef Islands	15	93	78[d]	53	20[d]
	total	120	92	71[a]	73	59[a]
Sri Lanka	Colombo area	10	100	100	100	90
Tanzania	Zanzibar	15	67	33	33	33
Thailand	1 Gulf of Thailand	45	100	100	93	71
	2 Phuket and surroundings	4	100	75	75	75
	total	49	100	98	92	71
Tonga	Tongatapu	25	96	96	72	52
Truk		2	50	50	50	50
Tuvalu	Funafuti	40	93	63	38	23
Vanuatu (a = only 21 assayed) (b = only 13 assayed)	1 Espiritu Santo (local)	18	78	85[b]	56	50[b]
	2 Efate (local)	4	100	50	75	25
	3 Tanna (local)	4	75	75	25	0
	total local	26	81		54	

Table 17.1 **(cont'd) Survey results for coconut samples (PAGE–hybridisation assay at high stringency using a CCCVd probe).**

Locations included in the survey			Percentage with CCCVd-related nucleic acids			
Country	Site	No. samples	Sample overall		Viroid region	
			Low stringency	High stringency	Low stringency	High stringency
	4 Saraoutou Station (introduced)	40	98	98	98	98
	total	66	91	76[a]	80	43[a]
Western Samoa	Upolu	26	100	100	58	42
Yap		2	50	50	50	50

Total number of countries: 29 (27 sampled for coconut)
Total number of sites: 50 (48 sampled for coconut)
Total number of coconut samples: 1024.

Figure 17.1. **Autoradiograph of PAGE–elecroblot–hybridisation assay of coconut samples from different areas (numbers below designate sampling sites). Each pair represents one sample following a low-stringency (left) and high-stringency (right) wash. Monomeric (246 nucleotides) and dimeric (492 nucleotides) CCCVd marker bands are indicated for each by a bar between each gel pair. TI: palm infected with CTiVd; CC: palm infected with CCCVd.**



Bands binding the probe and representing molecules with moderate sequence similarity to CCCVd, regardless of their structure (column labelled 'Sample overall, low stringency'), were found in coconuts at all sites, but they were not necessarily identical to each other. The incidence ranged between 50% and 100% (with an average of 91%). When these bands were scored according to their similarity in electrophoretic mobility to CCCVd (column labelled 'Viroid region, low stringency'), positives (i.e. moderate sequence similarity, high structural similarity) were found at all sites in the range 33% to 100% (with an average of 71%). In the class where all bands with strong sequence homology to CCCVd were scored regardless of their structure (column labelled 'Sample overall, high stringency'), positives were found at all sites in the range 33% to 100% (with an average of 79%). When only those bands representing molecules with relatively strong sequence homology to the probe and with electrophoretic mobility similar to CCCVd (column labelled 'Viroid region, high stringency') were scored,

positives (i.e. high sequence as well as structural similarity) were found in all but one site (47/48) in the range 17% to 100% (with an average of 56%).

Nucleotide sequencing must be carried out to provide information on whether some of these molecules can be classified as new viroids related to CCCVd and CTiVd.

The results can be interpreted to indicate that the molecules identified in the survey vary in sequence and structural similarity to CCCVd. The test used is designed to detect nucleic acids related to CCCVd, but further studies are needed to sequence, classify and characterise the various molecules detected in this survey, and to assess the risk they pose to the countries. In the long term, their infectivity and pathogenicity need to be tested.

Tables 17.2a–d provide comparisons of countries and sites according to both the number of samples collected there (Tables 17.2a and c), and the percentage of samples found to contain molecules closely related to CCCVd (Tables 17.2b and d; corresponding to the column labelled 'Viroid region, high stringency' in Table 17.1). Locations where fewer than 5 samples were taken are not included. Rankings given are entirely preliminary since they are based only on what was collected during a qualitative survey. The data are not sufficient to establish a link between geographic or ethnic factors and the presence of CCCVd-related sequences.

Table 17.2.Preliminary rankings of countries and sites by number of samples collected and number with probe-binding nucleic acids similar in size to CCCVd. (For Federated States of Micronesia see PLW, PON, TRU, YAP.)

Table 17.2a. Countries ranked by number of coconut samples collected.

Rank	Country	No. samples per country
1	PHL	153
2	SLB	120
3	FJI	105
4	AUS	71
5	VUT	66
6	PYF	64
7	THA	49
8	KIR	43
9	TUV	40
10	GUM	38
11	IDN	37
12	PNG	32
13	COK	31
14	NCL	29
15	MHL	28
16	WSM	26
17	TON	25
18	TZA	15
19	LKA, MYS	10
20	PON	9
21	NRU, CRI	6
22	MOZ	5
23	PLW, TRU, YAP	2
24	NZL, IND	0

Table 17.2b. Countries ranked by percentage of coconut samples containing nucleic acids binding to probe at high stringency and similar in size to CCCVd. Data included only when 5 or more samples were tested.

Rank	Country	% positive*
1	MYS, LKA	90
2	PON	89
3	PNG	85
4	THA	71
5	COK	68
6	CRI, FJI, NRU	67
7	NCL	62
8	SLB	59
9	AUS	54
10	TON	52
11	IDN	49
12	PYF	45
13	VUT	43
14	WSM, GUM, PHL	42
15	MOZ	40
16	KIR	37
17	TZA	33
18	MHL	25
19	TUV	23

* Percentage of samples showing bands in the viroid monomer region after washing at high stringency.

Table 17.2c. Sites ranked by number of coconut samples collected.

Rank	Site (country)	No. per site
1	Luzon (PHL)	78
2	Russell Islands (SLB)	68
3	Mindanao (PHL)	63
4	Far north Queensland (AUS)	56
5	Gulf of Thailand (THA)	45
6	Tarawa (KIR)	43
7	Saraoutou Station (VUT), Funafuti (TUV)	40
8	Guam (GUM)	38
9	Viti Levu (FJI)	32
10	Rotuma (FJI)	31
11	New Britain (PNG)	30
12	Majuro (MHL)	28
13	Tahiti (PYF), Upolu (WSM)	26
14	Tongatapu (TON), Guadalcanal	25
15	West Java (IDN)	24
16	Lakeba (FJI)	23
17	Rangiroa (PYF)	21
18	Vanua Levu and Taveuni (FJI)	19
19	Espiritu Santo (VUT)	18
20	Nuku Hiva (PYF), Rarotonga (PYF)	17
21	Aitutaki (COK), Nouméa (NCL)	16
22	Santa Cruz and Reef Islands (SLB), Zanzibar (TZA)	15
23	Ouvéa (NCL)	13
24	Mindoro (PHL), Rennell Island (SLB)	12
25	Selangor (MYS), Cocos (Keeling) Is (AUS), Colombo area	10
26	(LKA)	9
27	Ponape (PON)	7
28	Central Kalimantan (IDN)	6
29	North Sumatra (IDN), Nauru (NRU), Atlantic Zone (CRI)	5
30	Groote Eylandt (AUS), Zambesia province (MOZ)	4
31	Efate (VUT), Tanna (VUT), Phuket (THA)	2
32	Yap (YAP), Truk (TRU), Palau (PLW), Port Moresby (PNG)	0
	North Island (NZL), Kerala (IND)	

* Percentage of samples showing bands in the viroid monomer region after washing at high stringency.

Symptoms, Host Parameters and CCCVd-Related Molecules

For each sample, photographic records were taken (Figs 17.2a–h), and observations were made on the presence of possible symptoms and their nature, together with other specific details such as palm origin, cultivar, age, and environmental factors (Chapter 15, appendix 15.3).

The statistical analysis of these data in relation to the presence and type of CCCVd-related molecules was kindly provided by L. Giles and T. Hancock, Department of Biometry, University of Adelaide. Using Genstat, gel banding patterns were tabulated against the selected characteristics. We were interested in whether the presence of CCCVd-related molecules and symptoms are independent or associated. In every instance the relationship between the palm characteristics and the bands appeared to be independent, thus no specific tree characteristics could be shown to be associated with the presence of CCCVd-related molecules (Sokal and Rohlf 1969).

Figure 17.2. **Examples of palms from various countries containing CCCVd-related molecules and showing a variety of symptoms.**

Figure 17.2a. **Ruffled crown, spindle-shaped nuts.**

Figure 17.2b. **Yellow crown, no nuts.**

Figure 17.2c. Frond yellowing, reduced bearing.

Figure 17.2d. Few nuts, necrosis of inflorescences.

Figure 17.2e. No abnormalities.

Figure 17.2f. Ruffled crown, not bearing.

Figure 17.2g. **Reduced crown, frond yellowing, no nuts, stem tapering.**

Figure 17.2h. **Reduced crown, short fronds, chlorosis, no nuts, tapered trunk. no nuts, stem tapering.**

18 CCCVd-RELATED SEQUENCES IN SPECIES OTHER THAN COCONUT

A. The detection of viroid-like sequences related to coconut cadang-cadang viroid in oil palm (*Elaeis guineensis*) with preliminary evidence for both horizontal and vertical transmission

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Introduction

The unexpected detection of CCCVd-related molecules in coconut and oil palm in Solomon Islands led to a widespread survey of countries in the Pacific under ACIAR sponsorship (Hanold and Randles 1991b). The viroid-like molecules in coconut differed from CCCVd in both size and sequence, but were nevertheless related to CCCVd on the basis of the Northern blot results obtained (Chapter 17). Not all coconut palms contained the sequences, and no palms showed typical cadang-cadang disease, although recognition of the disease in isolated palms by symptoms alone is difficult and unreliable. However, a number of oil palms with CCCVd-related sequences showed orange leaf spotting, suggesting that this may be a symptom of infection with a viroid related to CCCVd.

Part A of this Chapter briefly outlines the background to the detection of CCCVd-related nucleic acid molecules in commercially grown oil palm in SE Asia and the South Pacific. It summarises observations of the association of the sequences with symptoms and growth of oil palms in the field. Preliminary evidence for spread in the field is presented, together with data that suggest that yield declines with time in commercial plantations. The origin of commercial germplasm is outlined and the possibility of vertical transmission is discussed. Suggestions are made for a research program to evaluate the importance of these viroid-like sequences.

Oil palm and CCCVd

Oil palms grown at the Albay Research Center during the 1970s showed orange spotting of leaflets and a decline resembling cadang-cadang disease in adjacent coconut palms. CCCVd was recovered by PAGE assay and molecular hybridisation (Randles et al. 1980). Seedlings that were inoculated with CCCVd by the high pressure injection method showed yellow spotting symptoms 1–2 years later; this was associated with the detection of CCCVd (Imperial et al. 1985).

I observed an oil palm in a commercial plantation in Solomon Islands in 1986 with yellow leaf-spotting resembling that found in CCCVd-inoculated oil palms in the Philippines. A CCCVd-related molecule was detected in the palm (Hanold and Randles 1991b). Further studies showed that palms with the yellow spotting, which was characteristic of genetic orange spotting (GOS) (Turner, pers. comm.), contained sequences related to CCCVd by size, structure and sequence.

Some symptomless palms also contained the viroid-like sequences (Hanold and Randles 1991b).

Orange spotting is a well-known disorder of oil palm (Coulter and Rosenquist 1955; Smilde 1962, 1963; Robertson et al. 1968; Forde and Leyritz 1968). It has been variously described as confluent orange spotting, orange spotting and genetic orange spotting. A distinction has been made between the symptoms characteristic of confluent orange spotting and genetic orange spotting. The former is associated with nutrient deficiencies. However, the heritability of genetic orange spotting, its association with particular progenies, the lack of association with major nutrient deficiencies, and the absence of necrosis from the spots led the above authors to the hypothesis that the disorder was inherited. This would imply that it has neither a biotic nor an abiotic cause. The symptoms observed in oil palms in this work closely resemble those of genetic orange spotting (Turner 1981), and GOS is the term used in this Chapter to describe the typical orange spotting up to about 5 mm in diameter observed on more than half the fronds of the palms.

The consistent isolation of a CCCVd-related sequence from oil palm with GOS has provided an alternative hypothesis to the genetic hypothesis: that is, that GOS is a seed-transmissible disease caused by a viroid with a sequence similar to but different from CCCVd. For simplicity, this viroid is tentatively named oil palm spotting viroid (OSVd), but purification and sequencing have yet to be done to determine its properties.

Some features of GOS-affected palms are discussed here.

Effects on Growth

Forde and Leyritz (1968) reported around a 50% decline in yield of bunches from GOS-affected palms compared with adjacent healthy palms. About 93 palms in an 8-year-old commercial plantation in Solomon Islands that had been assayed for OSVd were measured for height of trunk from ground level to the lowest bunch of fruits. As shown in Table 18A.1, the palms with OSVd alone showed some stunting compared with sequence-negative palms and palms containing molecules of low sequence homology to CCCVd, but palms with both OSVd sequences and GOS showed significant stunting.

Table 18A.1. Relationship between trunk height of oil palms, the presence of OSVd and the incidence of GOS in a selected area of 93 trees in a plantation in Solomon Islands. (Solomon Islands Plantations Ltd, block 64, planted 1983; viroid assay 1989; height measured 1991.)

Viroid band status	No. trees	GOS symptoms	Mean tree height (cm) ± SE
No bands	9	no	312 ± 17
Bands of low homology to CCCVd	5	no	303 ± 35
Bands of high homology to CCCVd	70	no	290 ± 8
Bands of high homology to CCCVd	9	yes	217 ± 12

Relationship between Presence of Symptoms and Detection of OSVd

The incidence of OSVd was determined by testing trees in a commercial block of oil palms at 6 years after planting (Table 18A.1). Leaf extracts were assayed by Northern blots as described in Chapter 16, and the strength of the signal was estimated after a low-stringency wash. Sixty-seven per cent of the palms showed a strong positive signal, 22.3% showed a weak positive signal, and 10.8% were negative. The association between OSVd and the presence of GOS is shown in Table 18A.2. GOS was classed as either severe (over 75% of the crown affected) or mild (orange spotting on the lower whorls of fronds only). OSVd was commonly associated with GOS, but OSVd also occurred in the absence of spotting. In 1 case no OSVd was detected in a severely affected GOS palm, and in 10 cases no OSVd was found in mildly affected GOS palms (Table 18A.2).

Table 18A.2. Relationship between the incidence of GOS and the detection of OSVd in oil palms in block 64, SIPL, Solomon Islands.

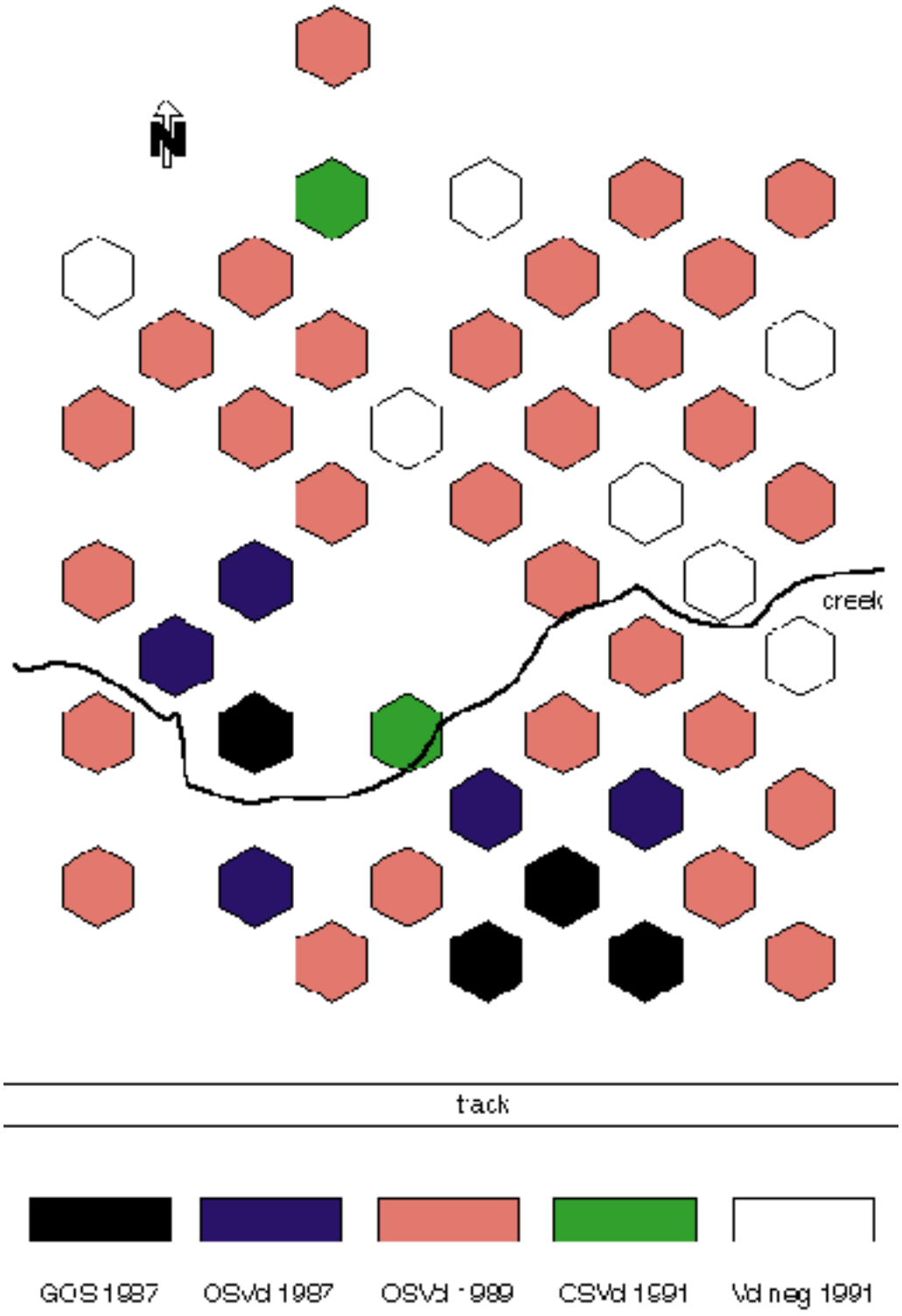
OSVd signal	Severe GOS	Mild GOS	Asymptomatic	Total
Strong	38	34	9	81
Weak	7	18	2	27
Nil	1	10	2	13
Total	46	62	13	121

Evidence for Transmissibility

Some evidence that OSVd is infectious is presented in Chapter 19, where coconuts inoculated with an extract from a GOS-affected oil palm developed a viroid-like band 12 months after inoculation.

To determine whether natural spread of OSVd occurs in the field, the palms in the plot shown in figure 8 of Hanold and Randles (1991b) were resampled for assay for OSVd on 4 December 1989 and 9 February 1991. The first survey, in 1987, included 4 GOS palms that were all positive for OSVd (results of Hanold and Randles 1991b). The second survey, in 1988, was of 14 palms, including the 4 GOS palms. Of these, 9 (64%) were positive for OSVd. In 1989 and 1991, 47 palms were sampled, and the incidence of OSVd was 81% and 85% respectivelyó2 new infections were detected between the 2 survey dates (Fig. 18A.1). Of the original 14 sampled in 1988, all were positive in 1991. This survey therefore provides strong evidence that natural spread of OSVd occurs.

Figure 18A.1. **Distribution of palms with OSVd in Block 27, SIPL, shown in relation to time of assay. Note increase in incidence with time and apparent gradient from a focus near the 4 palms with GOS.**



Inheritance of GOS

In a trial at Dodo Creek Research Station, Solomon Islands, by J. Saelea and R. Liloquila, seednuts collected from 2 oil palms with GOS were germinated in a screenhouse (Fig. 18A.2). At 13 months after germination, 4/18 of the progeny from 1 palm and 2/12 of the other showed GOS. This showed that GOS was seed-transmissible, in agreement with earlier reports from other countries, and that it was apparent within 13 months of germination.

A survey of a 3-year-old planting of oil palm near Medan, North Sumatra, on newly cleared land, showed an incidence of GOS in the range of 1%–2%. Detection at such an early stage in the field would be expected to be due to seed-transmission of the symptom. Four of the GOS seedlings were assayed, and 2 contained OSVd. Two symptomless seedlings assayed also contained OSVd. The interpretation of these results will require assay with OSVd specific sequences once they become available. However, one interpretation could be that the GOS palms act as a primary focus for OSVd, and that secondary spread to other palms in the plantation occurs from these foci.

To test the possibility that spread occurs in the field, resulting in a decline in production, gross yields for the period 1985 to 1989 were obtained for the Sawit Seberang plantation near Medan. As shown in Table 18A.3, yield declined by about 25% over this period, from about 23 to 17 t/ha. This was not explained adequately by agronomic factors. Weevils introduced to aid pollination in 1983 may have had a direct deleterious effect on production, but there is also the possibility that their foraging and feeding could transmit an agent such as a viroid that could be causing reduced production. Frequent mechanical harvesting with scythes may also lead to mechanical inoculation within the plantation.

Table 18A.3. Yield regression in annual oil palm production (tonnes of bunches/ha/y) in Sumatra.

Year	Area planted (ha)	Yield (t/ha)	% of 1985 yield*
1985	5301	23.662	100
1986	5927	20.988	88.7
1987	5925	17.647	74.6
1988	6181	18.918	79.9
1989	6758	17.816	75.5

* Weevils were released for pollination in 1983.

Possible Vertical Transmission of GOS and OSVd

GOS has been observed in commercial oil palm plantations in West Africa, Indonesia, Malaysia, Thailand, Papua New Guinea, Solomon Islands and Central America. Moreover, samples of oil palm from South America contained OSVd (Hanold and Randles 1991b). Oil palm has a very narrow genetic base, as shown in Table 18A.4.

The widespread distribution of GOS and OSVd could therefore be readily explained by the vertical transmission of the causal agents and their distribution with seed.

As shown in Table 18A.4, recently planted, commercial oil palm plantations are derived from 4 parents. For example, oil palms with GOS in the Solomons are descendants of parents in a seed garden in Papua New Guinea, which in turn derived its germplasm from Malaysia. Malaysian material was derived from Sumatran Deli dura palms.

Figure 18A.2. 'Genetic' orange spotting (GOS) syndrome of oil palm.

Figure 18A.2a. Oil palm with GOS symptoms.

Figure 18A.2b. Leaflets from fronds of increasing age (left to right) from a GOS tree. Note increasing frequency and size of spotting with age of frond, and the distal necrosis on the oldest leaflet.

Figure 18A.2c. Crown of GOS palm showing stunting compared with neighbouring trees of the same age (below).

Figure 18A.2d. Distal part of frond of GOS palm.

The parents were *pisifera* male from Sumatra (SP540) and Deli *dura* in the Bogor Botanic Garden, Indonesia (one of which was living in 1990, aged about 148 years). Currently growing West African and South American commercial oil palm plantations are also derived from Sumatran material.

The following observations are consistent with the possibility that vertical transmission of GOS and OSVd has occurred.

- The 1 survivor from the original parental material of the Bogor *dura* parents shows evidence of bronzing of older fronds. Leaf samples could not be taken for assay, but a root-tip assay identified an OSVd-like band.
- One 10-year-old palm that was the progeny of the *dura* survivor growing at the Bogor Botanic Garden was OSVd-positive.
- Twenty-four seedling progeny of the Bogor *dura* parent, planted in 1976 at Sungei Pancur, Sumatra, were assayed. Three showed GOS and were also positive for OSVd, 9 were strongly positive for OSVd, and 11 were weakly positive for OSVd.
- *Pisifera* SP540 planted in 1923 at Sungei Pancur as the pollen source for early *dura* × *pisifera* crosses had an OSVd-like band in root-tip samples.
- Selfing of a symptomless Deli *dura* oil palm selection (L269D) in the IRHO program in Côte d'Ivoire yielded progeny seedlings with over 90% orange spotting. This segregation does not follow a pattern typical of genetic inheritance, and cannot be explained. This expression in the F1 generation could be due to a pathogen such as OSVd being seed-borne, in a manner similar to that described for avocado sunblotch viroid (Desjardins 1987). Other progeny of L269D are reported to show high incidence of orange spotting in Africa and SE Asia.

Table 18A.4. History of the commercial development of African oil palm. (Derived from Hartley 1977; IRHO, Paris; Dami Oil Palm Research Station, PNG; and personal communications.)

Time	Place	Notes
26–7m y b.p.	Niger delta	Oil palm pollen in Miocene deposits
1400s	to Brazil	With slave trade from Africa
1456	Guinea coast	Recorded
1506	Liberia, Nigeria	Recorded
1696	to Jamaica	From Guinea
	to East Africa and Madagascar	From West Africa and Zaire
1830	West Africa	First exports of palm oil
1848	Botanic Garden, Bogor, Java	Parental seedlings planted: 2 from Mauritius or Réunion, 2 from Africa via Amsterdam
1875	Deli, Sumatra	*Dura* seedlings ex Bogor
1884	Sumatra	Avenues planted
1911	Sumatra	First commercial Deli *dura* plantings
1912	Selangor, Malaysia	Deli *dura* planted
1920	French West African territories, Zaire, Nigeria	Deli *dura* imported for comparison with local *tenera*. Commercial plantings included *dura*.
1922	Zaire	Djongo *tenera* selection
	Sumatra	SP540 *tenera* planted ex Djongo, SP2041 *dura* selected
1928	Ivory Coast	D115D *dura* introduced from Deli
1965	Ivory Coast	*Dura* × *tenera* hybrids developed. Widespread planting of hybrids began in many countries; continues to the present.
1968	Papua New Guinea	Deli *dura* and *pisifera* parents from Malaysia, for hybrid seed production
1973	Solomon Islands	Planting of seed from Malaysia and PNG
1990	Bogor, Java	One *dura* parent survives
	Sungei Pancur, Sumatra	One SP540 *pisifera* parent survives

Conclusions and Future Directions

The observations made on the phenomenon of GOS, and the apparent association between GOS and the detection of CCCVd-related sequences (OSVd) in oil palms, provide a viroid hypothesis for GOS. Other hypotheses suggesting either nutritional or genetic causes are not supported by the evidence. However, the detection of similar sequences in non-spotted oil palms suggests that OSVd may be presymptomatic, or that overt leaf symptoms are not always produced in oil palm.

A possible analogy can be drawn with avocado sunblotch viroid (ASBVd), where symptomatic avocado trees transmit the disease to a low (5%) proportion of seedlings, but where asymptomatic trees show 90%–100% transmission of viroid to seedlings (Desjardins 1987). If OSVd has a similar relationship with the host, the GOS palms may be analogous to the symptomatic ASBVd infections. This could explain the seed transmission described above. The large number of symptomless OSVd-positive palms may be analogous to the symptomless carrier condition between ASBVd and its host. For example, symptomless ASBVd infections can act as a reservoir for infection by both mechanical and pollen transmission, and there is a reduction in yield despite the absence of symptoms. The symptomless OSVd-positive oil palms may act as sources of infection via either mechanical or pollen transmission, as well as providing OSVd positive seed to the next generation and reducing yield.

The observations presented support the need for further research of both OSVd and GOS, particularly because there is a possibility that infected seed is being widely used for the extensive plantings of oil palm in many tropical countries. The immediate need is to isolate, sequence and compare the CCCVd-related molecules (representing OSVd) that occur in oil palm. Probes resulting from the sequencing study would allow pathology, epidemiology and control strategies to be examined. The incidence, transmissibility and effects on yield either alone or in combination with other parasites and pathogens can be tested once a sensitive molecular test has been developed.

In summary, the following steps need to be carried out:

1. Isolate, purify and clone isolates of OSVd.
2. Develop molecular probes for OSVd.
3. Sequence OSVd and compare with other viroids.
4. Inoculate and test the infectivity of OSVd clones.
5. Evaluate the effect of OSVd on oil palm productivity.
6. Study the epidemiology of OSVd with specific probes to determine the rate, pattern, range and timing of spread.

Acknowledgments

To Dr D. Hanold for assays and interpretation of results, to ACIAR for funding, and to Ir. C. Utomo for assistance in the survey in Medan.

18 CCCVd-RELATED SEQUENCES IN SPECIES OTHER THAN COCONUT

B. Survey results for species other than coconut and oil palm

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A total of 466 samples of species other than coconut and oil palm were also collected from most countries and analysed, including 89 other species of palms, 138 Pandanaceae, 105 Zingiberaceae, 17 Marantaceae, 46 Commeliniflorae and 91 monocotyledons of other families. The samples were classed into these 6 groups, because sample numbers of individual species were mostly small. Details of the results are shown in Table 18B.1, and examples of autoradiographs are given in Figure 18B.1. Some of the samples also contained CCCVd related molecules, which need to be compared with the coconut isolates to determine whether the other species could serve as reservoirs. None of these species showed any symptoms (Fig. 18B.2a–k). One of the hypotheses on the natural spread of CCCVd in the Philippines assumed that monocotyledonous herbaceous plants growing in association with coconut palms may act as latent reservoir hosts from which the viroid could be transmitted to the palms. So far, this hypothesis can be neither proved nor dismissed. Thus, sequencing of our collected herbaceous isolates and comparison with associated coconut isolates may help to clarify the situation and elucidate the mechanisms of CCCVd epidemiology.

Table 18B.1 Survey results for species other than coconut or oil palm, showing the frequency of detection of bands binding probe homologous to CCCVd. (LS = low-stringency post-hybridisation wash; HS = high-stringency post-hybridisation wash (see Chapter 17 for details). For Federated States of Micronesia see PLW, PON, TRU, YAP.)

Country	Other palm species					Pandanaceae					Zingiberaceae				
	Total no.	Positive with CCCVd probe				Total no.	Positive with CCCVd probe				Total no.	Positive with CCCVd probe			
		Overall		Viroid region			Overall		Viroid region			Overall		Viroid region	
		LS	HS	LS	HS		LS	HS	LS	HS		LS	HS	LS	HS
AUS	41	20	15	9	6	20	11	8	11	6	9	7	7	3	2
COK	0	-	-	-	-	4	4	4	4	4	2	2	2	2	1
CRI	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
FJI	6	3	3	2	2	24	24	23	24	21	24	11	8	5	5
GUM	6	2	2	2	1	2	2	2	2	2	0	-	-	-	-
IDN	0	-	-	-	-	2	2	2	2	2	1	1	1	1	1
IND	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
KIR	0	-	-	-	-	10	10	10	10	9	0	-	-	-	-
LKA	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
MHL	0	-	-	-	-	9	9	9	9	8	0	-	-	-	-
MOZ	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
MYS	0	-	-	-	-	1	1	1	1	1	2	2	1	2	1
NCL	1	0	0	0	0	0	-	-	-	-	0	-	-	-	-

Table 18B.1 (cont'd) Survey results for species other than coconut or oil palm, showing the frequency of detection of bands binding probe homologous to CCCVd. (LS = low-stringency post-hybridisation wash; HS = high-stringency post-hybridisation wash (see Chapter 17 for details). For Federated States of Micronesia see PLW, PON, TRU, YAP.)

NRU	1	1	1	1	1	1	1	1	1	1	0	-	-	-	-
NZL	1	1	1	1	1	0	-	-	-	-	0	-	-	-	-
PHL	2	2	2	2	2	1	1	1	1	1	2	2	0	1	0
PLW	3	0	0	0	0	0	-	-	-	-	0	-	-	-	-
PNG	0	-	-	-	-	2	2	2	2	2	15	14	8	11	3
PON	1	1	1	1	1	2	1	1	1	1	0	-	-	-	-
PYF	5	4	4	4	4	14	14	14	13	11	11	5	2	1	1
SLB	8	6	4	6	3	2	2	2	2	2	18	12	6	7	3
THA	0	-	-	-	-	1	0	0	0	0	2	2	1	1	0
TON	0	-	-	-	-	6	6	6	5	4	4	3	1	1	1
TRU	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
TUV	0	-	-	-	-	10	10	10	10	6	0	-	-	-	-
TZA	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
VUT	13	4	4	3	3	20	15	15	15	15	5	0	0	0	0
WSM	0	-	-	-	-	7	7	7	7	7	10	8	8	4	3
YAP	1	0	0	0	0	0	-	-	-	-	0	-	-	-	-
Total	89					138					105				

Table 18B.1 Survey results for species other than coconut or oil palm, showing the frequency of detection of bands binding probe homologous to CCCVd. (LS = low-stringency post-hybridisation wash; HS = high-stringency post-hybridisation wash (see Chapter 17 for details). For Federated States of Micronesia see PLW, PON, TRU, YAP.)

Country	Marantaceae					Commeliniflorae					Other monocots				
	Total no.	Positive with CCCVd probe				Total no.	Positive with CCCVd probe				Total no.	Positive with CCCVd probe			
		Overall		Viroid region			Overall		Viroid region			Overall		Viroid region	
		LS	HS	LS	HS		LS	HS	LS	HS		LS	HS	LS	HS
AUS	3	3	3	2	2	11	8	8	6	6	24	8	7	5	4
COK	1	1	1	0	0	1	1	1	1	0	5	5	5	3	3
CRI	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
FJI	3	3	3	2	2	2	2	2	2	2	11	9	8	5	3
GUM	0	-	-	-	-	4	2	2	2	0	1	1	1	1	1
IDN	3	3	3	3	2	0	-	-	-	-	2	1	1	1	1
IND	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
KIR	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
LKA	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
MHL	0	-	-	-	-	1	1	0	1	0	0	-	-	-	-
MOZ	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
MYS	1	1	1	1	0	0	-	-	-	-	4	3	3	3	3
NCL	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
NRU	0	-	-	-	-	0	-	-	-	-	1	1	0	0	0
NZL	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
PHL	2	2	2	2	2	2	2	0	2	0	1	1	0	1	0
PLW	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-

Table 18B.1 (cont'd) Survey results for species other than coconut or oil palm, showing the frequency of detection of bands binding probe homologous to CCCVd. (LS = low-stringency post-hybridisation wash; HS = high-stringency post-hybridisation wash (see Chapter 17 for details). For Federated States of Micronesia see PLW, PON, TRU, YAP.)

Country	Marantaceae					Commeliniflorae					Other monocots				
	Total no.	Positive with CCCVd probe				Total no.	Positive with CCCVd probe				Total no.	Positive with CCCVd probe			
		Overall		Viroid region			Overall		Viroid region			Overall		Viroid region	
PNG	2	2	2	2	1	3	3	2	3	2	0	-	-	-	-
PON	0	-	-	-	-	0	-	-	-	-	2	2	2	2	2
PYF	1	1	1	1	1	7	7	4	3	2	12	10	10	7	6
SLB	0	-	-	-	-	8	8	6	6	3	8	7	7	7	7
THA	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
TON	0	-	-	-	-	1	1	1	1	1	6	6	5	5	3
TRU	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
TUV	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
TZA	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
VUT	1	0	0	0	0	4	0	0	0	0	13	6	6	5	5
WS M	0	-	-	-	-	2	1	0	1	0	1	1	1	1	1
YAP	0	-	-	-	-	0	-	-	-	-	0	-	-	-	-
Total	17					46					91				

Figure 18B.1. Autoradiograph of PAGE–electroblot–hybridisation assay of samples of different species from various countries (numbers below designate origin of sample) after low-stringency post-hybridisation wash. Monomeric (246 nucleotides) and dimeric (492 nucleotides) CCCVd marker bands are indicated on the left. CC: palm infected with CCCVd; TI: palm infected with CTiVd. Cn: *Cocos nucifera*; Eg: *Elaeis guineensis*; As: *Alpinia* sp.; Ps: *Pandanus* sp.; Ms: *Maranta* sp.; Ac: *Areca catechu*.

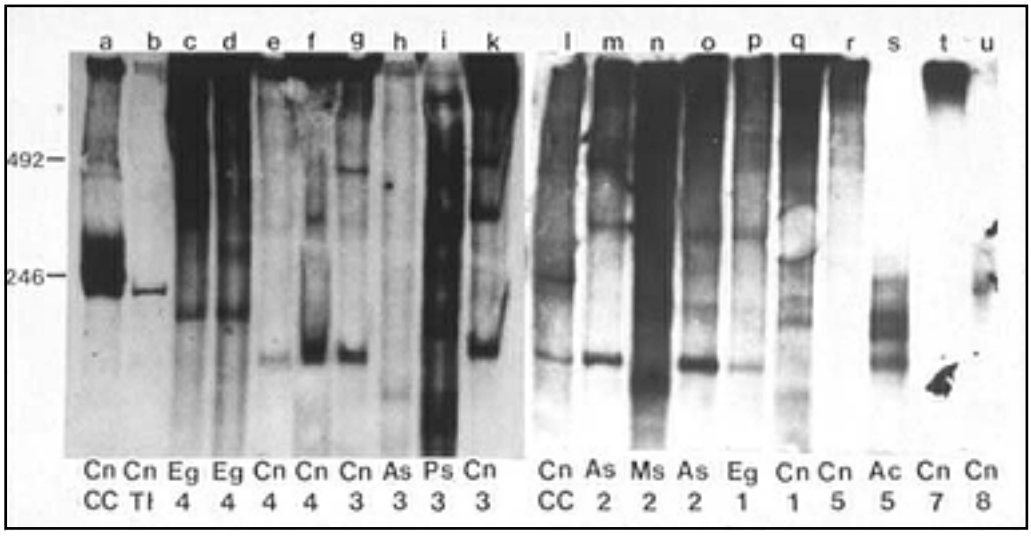

Figure 18B.2.Examples of other species collected.

Figure 18B.2a. ***Licuala*** **sp.**

Figure 18B.2b. ***Pelagodoxa henryi***

Figure 18B.2c ***Pandanus*** **sp.**

Figure 18B.2 d. ***Pandanus*** **sp.**

Figure 18B.2e. ***Maranta*** **sp.**

Figure 18B.2 f ***Alpinia*** **sp.**

Figure 18B.2g ***Alpinia*** **sp.**

Figure 18B.2 h ***Alpinia*** **sp.**

Figure 18B.2k. A species in the Commeliniflorae

Figure 18B.2i. A species in the Zingiberaceae

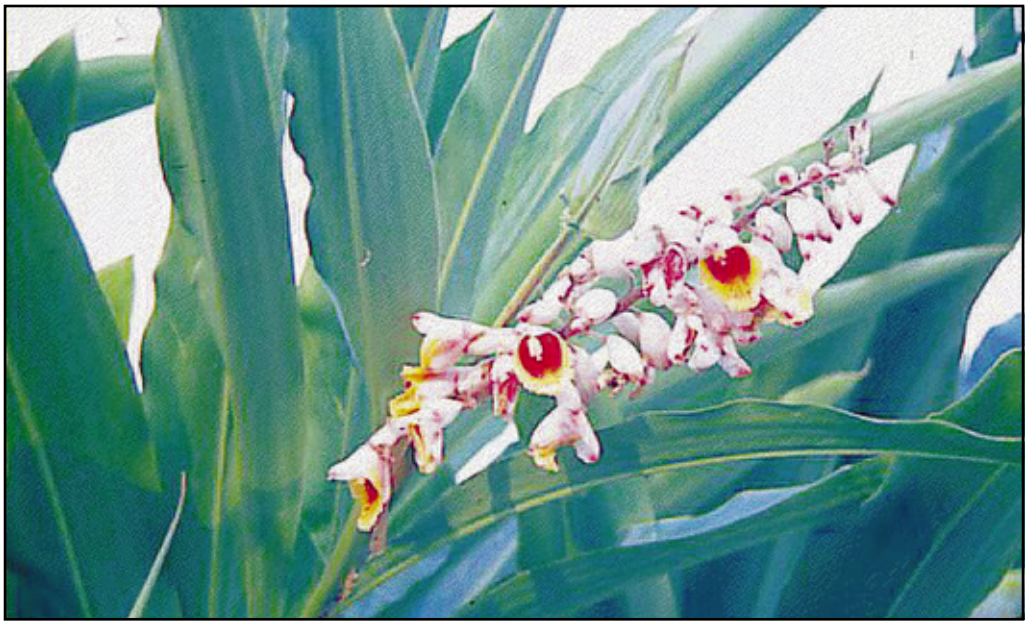

Figure 18B.2j. *Commelina* sp.

19 INVESTIGATION OF THE CHARACTERISTICS OF THE CCCVd-RELATED MOLECULES

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Molecular Structure

To determine whether the CCCVd-related sequences in samples collected outside the Philippine cadang-cadang areas were associated with viroid-like molecules, several variations of denaturing PAGE were used. Diagnostic tests for viroids are often based on PAGE under both non-denaturing and denaturing conditions because of the characteristic change of behaviour of these molecules during comparison of one set of conditions with the other.

A total of 15 samples previously found to contain viroid-like nucleic acids were comparatively analysed by 10% non-denaturing and denaturing PAGE systems, and the CCCVd-related bands were detected by hybridisation. The samples included 5 coconuts, 2 African oil palms, 2 Pandanus and 6 other monocotyledons from 8 countries.

In this system, CCCVd shows 2 bands under native conditions and 1 band in the denaturing gel. The aim, therefore, was to attempt to identify bands in the other samples that showed similar behaviour, which indicated a molecular structure similar to CCCVd. A variety of banding patterns resulted from both gel systems. When these were compared with purified CCCVd patterns, interpretation of the results was difficult due mainly to the numerous bands in the tracks, which made cross-identification in the denaturing and non-denaturing gels impossible. In several instances, a larger number of bands were present in the denaturing gel; this could be explained by assuming that denaturing conditions would allow species of molecules to enter the gel that were excluded in their natural form, owing to their size, complex structure or adhering impurities. However, without separate purification and analysis of each band, no firm conclusions could be drawn.

Further investigation of the molecular structure was therefore done using a 5% non denaturing/ denaturing 2-dimensional PAGE system followed by electroblot–hybridisation assay.

Table 19.1 shows the results of this analysis. Thirty-two samples previously found to contain CCCVd-related sequences were chosen from a range of species and locations, including 3 coconuts, 1 oil palm, 7 other palms, 3 Pandanaceae and 18 other monocotyledons from 11 countries. Most samples could be analysed only at low stringency, because the very limited amount of material still available for this additional test made it necessary to expose autoradiographs for several weeks to obtain a readable signal. Thus a second exposure after a high-stringency wash was no longer feasible. Stripping and re-probing of membranes would have been required to allow an immediate high-stringency wash with subsequent exposure, but project funding ceased before this work could be done. However, keep in mind that all samples analysed in this way were selected in the first place because they were previously shown to contain molecules closely related to CCCVd in both structure and nucleotide sequence when they were assayed in our standard system at high stringency. Twenty-four of the samples, from

a range of species, were found to show a signal in a position expected for circular viroid molecules (Table 19.1). Figures 19.1a–h show examples of 2 dimensional gels for different species. Signal spots in these gels must be considered to represent molecules with circular viroid-like structure because of their position ahead of the front of linear nucleic acids (see Chapter 2 for detailed explanation). Their exact degree of relatedness to CCCVd needs further evaluation by nucleotide sequence determination.

Oil palm also contained a viroid-like component; an attempt was made to compare this component with CCCVd. Since the oil palm isolate hybridised strongly with the CCCVd probe and retained signal strength at high stringency, a high level of relatedness seemed likely. Temperature gradient gel electrophoresis (TGGE) was used to visualise the melting pattern of the suspected oil palm viroid isolate. There was an indication of a characteristic, viroid-like, sigmoid melting curve, but the apparatus was not set up to read temperatures across the gradient, and hence it was not possible to show that the transition occurred at the same temperature as for CCCVd (data not shown). Experiments such as including an internal standard for comparison with the oil palm isolate could not be continued owing to cessation of project funding. A major constraint to this work was also the limited amount of oil palm isolate available. Future work will have to establish a large-scale purification method for the suspected oil palm viroid to supply sufficient material for sequencing and other studies.

Table 19.1. 2-D PAGE assay*—Detection of CCCVd-related molecules with viroid like structure from a range of host plants.

Host	Sample	Country	Detection of viroid-like molecules at	
			low stringency	high stringency†
Cocos nucifera	PH A3	PHL	+	+
	PH S09	PHL	–	–
	FPO 15	PYF	+	+
Elaeis guineensis (without GOS)	TO 22	PNG	+	–
Areca catechu	GJ 23	SLB	+	–
	P 21	PON	+	–
Metroxylon sagu	GJ 34	SLB	+	+
M. warburgii	VA 17	VUT	–	–
Pelagodoxa henryi	FPO 5	PYF	+	–
Phoenicophorum sp.	FJ 40	FJI	+	NA
Rattan palm	VA 26	VUT	+	NA
Cycas sp.	VA 12	VUT	+	NA
Pandanus sp.	TOG 35	TON	+	NA
	KOA	SLB	–	–
Freycinetia sp.	WSA 39	WSM	+	–
Cordyline sp.	FJ 231	FJI	+	–
Maranta sp.	A 23	AUS	+	NA
	TAE	PNG	–	–
Canna sp.	FJ 140	FJI	+	–

Table 19.1. (cont'd) 2-D PAGE assay*—Detection of CCCVd-related molecules with viroid like structure from a range of host plants.

Cucurma domestica	M 6f	MYS	+	NA
Taro	FPO 137	PYF	–	–
Swamp taro	P 25	PON	+	NA
Alpinia sp.	LP 7	SLB	+	NA
	FJ 126	FJI	+	NA
Zingiber sp.	PIB	SLB	+	NA
	M 2b	MYS	+	NA
Heliconia sp.	TOG 10	TON	–	–
Strelitzia sp.	TOG 27	TON	+	NA
	VA 23	VUT	+	NA
Bambusa sp.	TOG 9	TON	–	–
Paspalum sp.	S 50.2	SLB	+	–
Commelina sp.	FPO 112	PYF	–	–

* Two-dimensional polyacrylamide gel electrophoresis; see Chapter 17
†NA = not assayed

Figure 19.1. Samples containing CCCVd-related molecules from different species in 2 dimensional PAGE analysis and detected by electroblot–hybridisation assay, except for a (silver-stained gel). Arrows indicate viroid-like molecules.

Figure 19.1a. CCCVd marker. The arrow indicates the 246-nucleotide form.

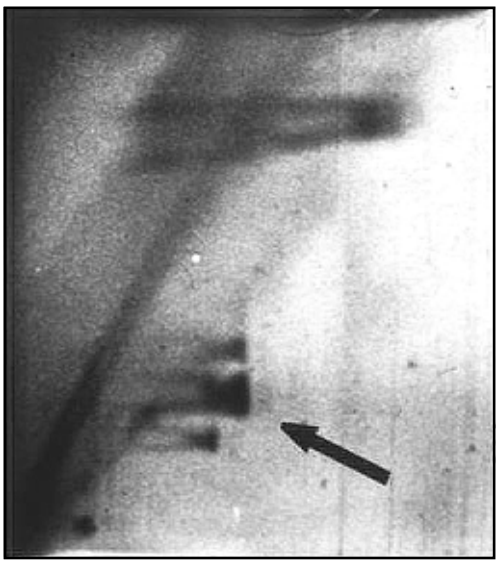


Figure 19.1b. Coconut palm from Solomon Islands (D) and healthy control (H).

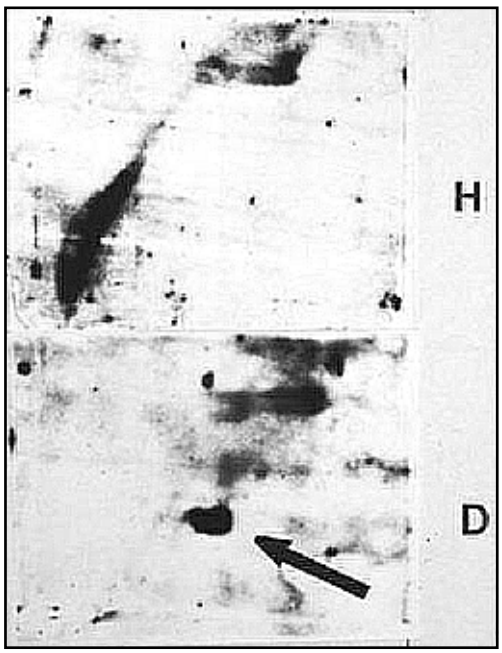


Figure 19.1c. **Coconut palm from Mindoro Island, Philippines.**

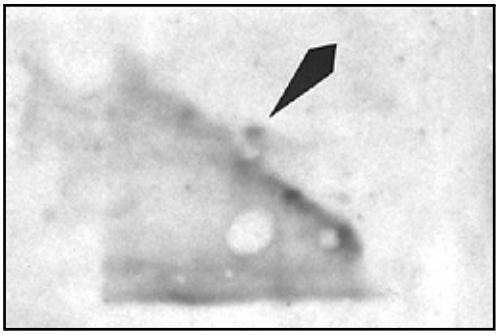


Figure 19.1e. ***Alpinia*** **sp., Fiji.**

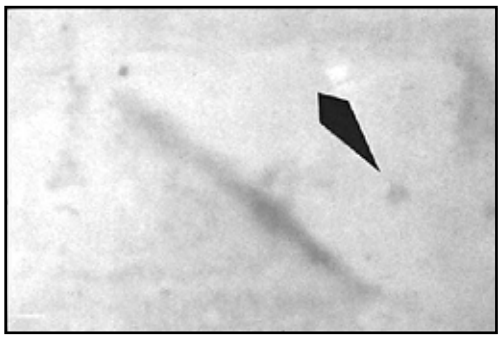


Figure 19.1f. ***Pandanus*** **sp., Tonga.**

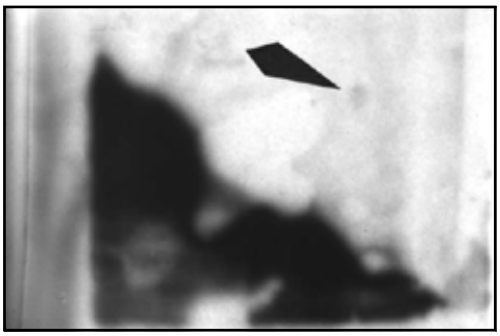


Figure 19.1g. ***Zingiber officinale*****, Malaysia.**

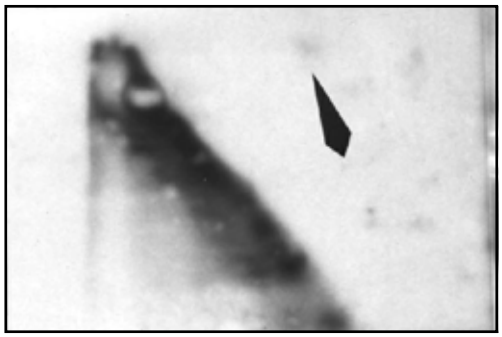


Figure 19.1d. **Healthy oil palm (H) and GOS palm (D).**

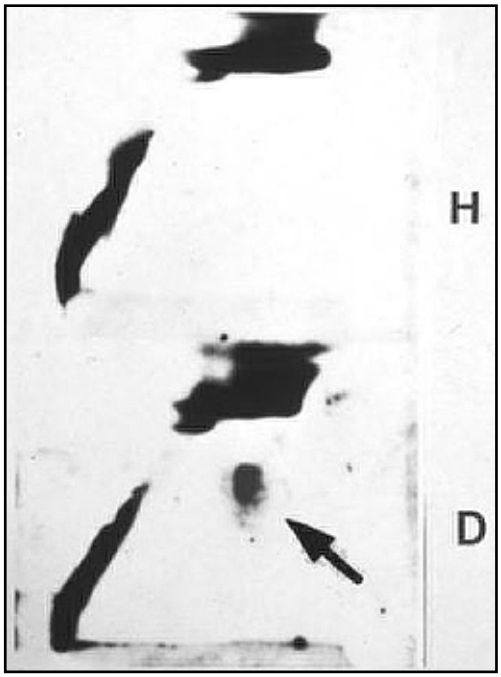
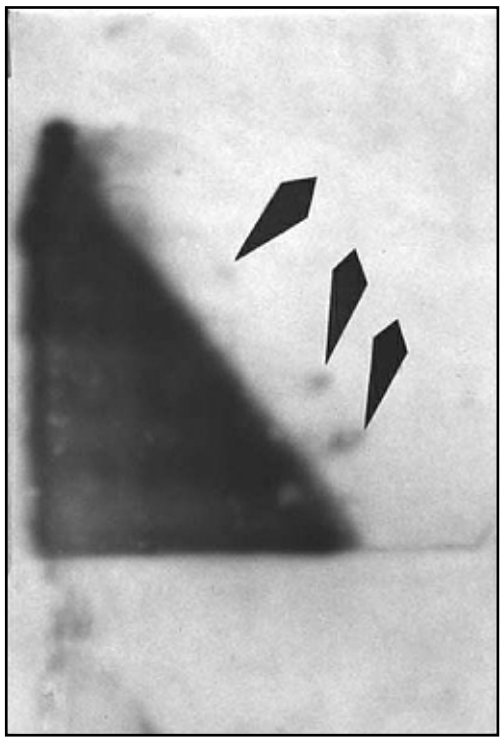


Figure 19.1h. ***Strelitzia*** **sp., Tonga.**



Purification

Methods devised for extracting CCCVd from coconut tissue were unsuitable for the other viroids because of their low concentration and the presence of contaminating material associated with other hosts.

Alternative strategies for obtaining sufficient viroid material of satisfactory purity for molecular studies had to be explored, and the oil palm viroid was chosen as a model system.

Amplification methods such as molecular cloning or the polymerase chain reaction (PCR) require pure starting material without enzyme inhibitors. Thus, as the initial step in the procedure for concentrating the viroid-like RNA, a 'fishing' technique was attempted.

A primer sequence complementary to the central conserved region of CCCVd was prepared for this purpose. In the absence of nucleotide sequence information, we considered that a closely related putative viroid would be most likely to share considerable sequence homology within this region (Koltunow and Rezaian 1989).

Oil palm isolate was extracted (see Chapter 17); the CTAB pellet was resuspended in 50% formamide, heated and snap-cooled; and hybridisation buffer was added at a ratio of 1 mL per 10 g of leaf starting material. Primer was dotted onto membranes and prehybridised, and 2 dots per mL of hybridisation suspension were immersed and hybridised at 37° without agitation for several days. The membranes were washed at very low stringency (45°C for 1 h in 1 × SSC, 0.1% SDS), rinsed in 1 × SSC several times to remove SDS, and boiled for 30 min in sterile distilled water. The water eluate was stored at –20°C. During boiling, the oil palm viroid attached to the filter-bound probe was expected to be released. The boiling process was repeated, the eluates were pooled, the volume was reduced by freeze-drying, carrier tRNA was added, and nucleic acids were precipitated with isopropanol in the presence of 0.1 M Na acetate. This preparation yielded a colourless pellet, which was used as a template for M-MuLV reverse transcriptase. However, only a small amount of cDNA was obtained.

An attempt was made to clone the cDNA by conventional methods, and some clones with inserts were obtained. However, these inserts had no sequence homology to CCCVd and thus the clones were discarded.

An attempt at amplification of the oil palm viroid cDNA by PCR using primers of the CCCVd conserved region yielded no detectable product.

Both of these procedures were preliminary and need to be tested again with suitable modifications.

As an alternative to the 'fishing', large-scale purification of the oil palm viroid by column PAGE was tried. Fractions containing viroid could be identified by dot blot hybridisation. It remains to be seen whether the nucleic acid product from this method is clean enough for enzymatic reactions, and can therefore be used in cloning or PCR.

Infectivity and Spread

Seed transmission

CCCVd appears to be seed-transmitted at a low rate in coconut. The oil palm 'genetic' orange spotting condition (GOS) received its name because early plantation work in Nigeria at NIFOR (Forde and Leyritz 1968) reported that its frequency in progeny depended on the particular parents involved in the cross. However, no true Mendelian distribution could be observed. Therefore, since we now suspect that a viroid could be the cause of GOS, its seed transmissibility seems a likely explanation of the African observations. To test this hypothesis, seeds from 2 GOS-affected oil palms were collected in Solomon Islands and germinated.

The progeny (Figs 19.2a, b) showed GOS at a rate of 4/18 and 2/12, respectively, corresponding to 20% seed-transmission overall.

Figure 19.2. Progeny of GOS oil palm.

Figure 19.2a. Seedling exhibiting orange spotting syndrome.

Figure 19.2b. Close-up of the distal part of one of the fronds (below). (Provided by J. Saelea and J. W. Randles.)

Inoculations

To establish whether the viroid-like molecules were infective, mechanical inoculation studies were done. Three experiments were set up (see Table 19.2):

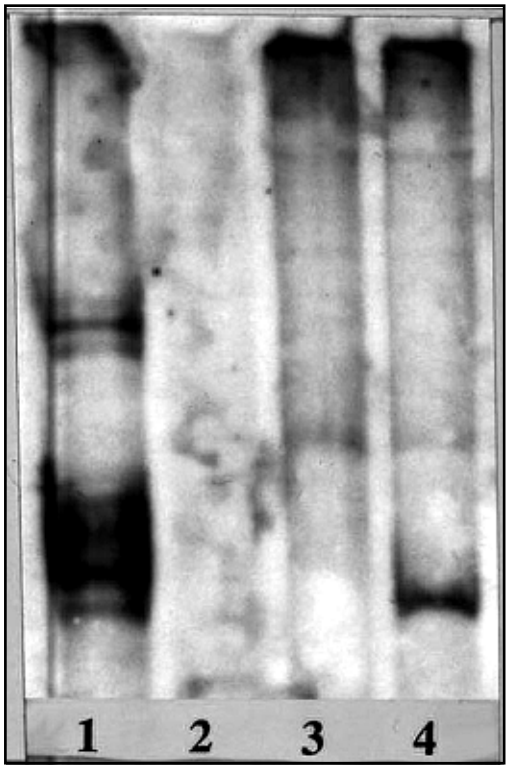
1. In the Philippines, coconut seedlings were inoculated with partially purified isolate from GOS-affected oil palm from Mindanao.
2. In PNG, both coconut and oil palm seedlings were inoculated with partially purified isolate from GOS-affected oil palm from New Britain.
3. In Adelaide, oil palm seedlings were inoculated with preparations from GOS-affected oil palm, and with a preparation from a coconut palm from Solomon Islands previously shown to contain a CCCVd-like molecule.

The inoculum for experiments 1 and 3 was prepared by $NaSO_3$ extraction with PVPP, $CHCl_3$ extraction, PEG precipitation, SDS–phenol–chloroform extraction and ethanol precipitation (see Chapter 16). Owing to the very limited facilities in the PNG laboratory, the inoculum for experiment 2 had no $CHCl_3$ extraction; protease digestion with SDS and dialysis was used instead of the phenol extraction; and final precipitation was done with CTAB rather than ethanol.

Assays were done using the system established for the survey (see Chapter 16), adjusted to a smaller scale of 1–2 g of leaf material.

In experiment 1, 2 out of 34 (5%) inoculated coconuts showed a single strong band in the CCCVd monomer region (Fig. 19.3). The inocula for these 2 plants originated from different GOS trees, and they were inoculated about 4 weeks apart and by a different person. In both cases, the corresponding uninoculated and mock-inoculated controls were negative. This strongly suggests that the pathogen causing GOS in oil palm is indeed an infectious viroid, and that its host range includes coconut as well as oil palm. None of the other experiments resulted in successful inoculations. However, varying levels of viroid-like nucleic acids were commonly detected for both inoculated and control seedlings from the same sources. At low stringency, this ‘background’ level was very high (98%) for Philippine coconuts, somewhat less (ca. 70%) for both oil palms and coconuts of PNG, and much lower (8%) for West African oil palms. This is consistent with the hypotheses that at least some members of the CCCVd family can be seed-transmitted, that multiple infections occur, and that the level of background, viroid-like sequences does not protect the coconuts from being infected with the GOS pathogen, since the 2 successfully inoculated plants had the background, viroid-like sequences as well, like the rest of the seedlings.

Figure 19.3. **Autoradiograph of PAGE–electroblot–hybridisation assay of coconuts inoculated with GOS oil palm extract. 1: CCCVd marker; 2, 3: inoculated coconut palms remaining negative for CCCVd-related molecules; 4: inoculated coconut palm showing a strong signal at the exact position of the 247 nucleotide form of CCCVd. Tracks 3 and 4 show some faint bands in non-CCCVd regions of the gel considered to be 'background' (see text for explanation).**



Sample Preservation

One of the main constraints for more extensive future surveys is the perishable nature of fresh leaf samples. This meant that so far we could include only locations with cold storage facilities and frequent air connections, so that samples could reach Adelaide within 1–2 weeks, being chilled to 4°C most of the time, to be in reasonable condition. Even so, occasionally samples were partly or totally spoilt owing to careless handling during transport. To avoid this pitfall, methods of preserving the tissue in the field with subsequent safe shipment were investigated.

Based on preliminary experiments done earlier in the Philippines with CCCVd-containing coconut leaf, 32 preservation treatments were assessed. Samples of 2 g of CCCVd containing leaf were cut into 1 cm pieces and subjected to the following procedures in duplicate.

Table 19.2. Results of inoculation experiments for the GOS agent and CCCVd-related molecules from coconut palm.

Inoculum	Inoculum preparation	Method of inoculation	Species inoculated	No. surviving test plants	Total leaf equivalent administered per plant	No. positive after ca. 1 year for CCCVd	% with background viroid-like sequences*
GOS oil palm (Mindanao)	Na_2SO_3PVPP, $CHCl_3$, PEG, SDS/ phenol, ethanol	3 d dark, several shots on 1 occasion 2–7 d after emergence	coconut	34/39	25 g	2 (5%)	98
buffer	–	3 d dark, several shots on one occasion 2–7 d after emergence	coconut	12/15	–	0	
none	–	–	coconut	13/14	–	0	
GOS oil palm (New Britain)	Na_2SO_3 , PVPP, PEG, SDS/protease, CTAB	7 d dark, 10 shots/ plant on 1 occasion at 2–3 leaf stage	oil palm	10/45	14 g	0	71
none	–	–	oil palm	24/58	–	0	
GOS oil palm (New Britain)	Na_2SO_3 , PVPP, PEG, SDS/protease, CTAB	2 d dark then as above, 2–7 d after emergence	coconut	33/36	14 g	0	72
none	–	–	coconut	24/33	–	0	
GOS oil palm (New Britain)	Na_2SO_3 , PVPP, PEG, SDS/protease, CTAB	No darkness, then as above 2–7 d after emergence	betelnut	1/1	14 g	0	0
none	–	–	betelnut	3/3	–	0	

Table 19.2. (cont'd) Results of inoculation experiments for the GOS agent and CCCVd-related molecules from coconut palm.

Inoculum	Inoculum preparation	Method of inoculation	Species inoculated	No. surviving test plants	Total leaf equivalent administered per plant	No. positive after ca. 1 year for CCCVd	% with background viroid-like sequences*
GOS oil palm (Guadalcanal)	Na_2SO_3 , PVPP, $CHCl_3$, PEG, SDS/ phenol, ethanol	At 2 leaf stage, 3 shots 3 × with 2–3 d intervals. 2–30 d later dark for 3 d then inoculation by abrasion. 7 d later several shots on 1 occasion	oil palm	51/56	55 g	0	8
Coconut with CCCVd-like molecules (Guadalcanal)	Na_2SO_3 , PVPP, $CHCl_3$, PEG, SDS/ phenol, ethanol	At 2 leaf stage, 3 shots 3 × with 2–3 d intervals. 2–30 d later dark for 3 d then inoculation by abrasion. 7 d later several shots on 1 occasion	oil palm	54/54	46 g	0	
none	–	–	oil palm	52/54	–	0	

I Drying

1. desiccation with silica gel at 4°C
2. heat drying (4 h at 60°–70°C)
3. heat drying with vacuum (2 h, 60°–70°C)
4. vacuum drying (8 h, approx. 20°C)

Long-term storage: sealed airtight with desiccant at ambient temperature.

II 1% SDS

1. boil 10 min
2. boil 1 min
3. 60°C 3 h
4. 60°C 20 min
5. soak at room temperature

Long-term storage: in treatment liquid at ambient temperature.

III 20% PEG 8000

1. soak at 4°C 1 week
2. soak at room temperature
3. degas 3 times in PEG

Long-term storage: in treatment liquid at ambient temperature.

IV Acetone

1. soak 2 d at 4°C, replace with fresh acetone, soak 1 week at room temperature, air-dry.

Long-term storage: sealed airtight with desiccant at ambient temperature.

V 5% TCA

1. vacuum infiltrate 3 times, then soak 2 d at room temperature
2. soak 2 d at room temperature

Long-term storage: in 1% TCA at ambient temperature.

VI Ethanol

1. soak in 96% ethanol at room temperature and replace with fresh ethanol after 1, 2, 4 h; soak overnight; air-dry
2. same as a, but no air-drying
3. soak in 96% ethanol at 60°C and replace after 30 min, 1 h, 3 h; soak overnight at room temperature; air-dry
4. same as c, but no air-drying
5. soak in 70% ethanol at room temperature

Long-term storage: for a and c, sealed air-tight with desiccant; for b, d and e, in treatment liquid; all at ambient temperature.

VII 3% glutaraldehyde in phosphate buffer pH 7.2

vacuum infiltrate 3 times, soak at 4°C for 2 weeks

Long-term storage: in 1% glutaraldehyde at ambient temperature.

VIII 3% formaldehyde (containing approx. 1% methanol)

1. soak at room temperature
2. vacuum infiltrate 3 times, soak at room temperature
3. boil 5 min, cool, and soak at room temperature.

Long-term storage: in treatment liquid at ambient temperature.

IX 'FAA' fixation (90 parts 70% ethanol, 5 parts glacial acetic acid, 5 parts 37% formaldehyde)

1. soak at room temperature
2. vacuum infiltrate 3 times, soak at room temperature

Long-term storage: in treatment liquid at ambient temperature.

X 50% glycerol

soak at room temperature

Long-term storage: in treatment liquid at ambient temperature.

XI 1% thioglycollic acid

1. soak at room temperature
2. vacuum infiltrate 3 times, soak at room temperature
3. boil 5 min, soak at room temperature

Long-term storage: in treatment liquid at ambient temperature.

XII 8 M urea

1. soak at room temperature
2. boil 5 min, soak at room temperature

Long-term storage: in treatment liquid at ambient temperature.

A first assay was carried out after 6 weeks by PAGE. Based on this, the best preservation method was judged to be acetone (IV), followed by ethanol (VI a–e) and vacuum drying (I d). For field collections, vacuum drying is not achievable, and acetone is a dangerous chemical that usually cannot be obtained in remote places and must not be carried by air. Therefore, an experiment was conducted with samples of coconut and Pandanus from Rotuma known to contain viroid-like molecules related to CCCVd. The experiment compared 2 preservation methods: dehydration for several days at ambient temperature in acetone (as control treatment), and an equal mixture of industrial alcohol and commercial car petrol (both usually available even in isolated places), and subsequent air-drying for transport sealed in plastic bags with desiccant.

The method using alcohol plus petrol was as successful as the acetone treatment, and was therefore adopted for the long-term preservation and storage of all survey samples.

20 CONCLUSIONS FROM THE SURVEY RESULTS AND SUGGESTIONS FOR FUTURE WORK

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After preliminary results were made available to all countries on a confidential basis, a questionnaire (Appendix 20.1) was handed out to the participants at a meeting organised by the South Pacific Commission (SPC) in Suva in September 1991 to assist with planning of the follow-up work required. The responses are summarised in Table 20.1. They confirm that all coconut-growing countries place very high importance on the improvement of their local germplasm, be it for reasons of increasing the general nutritional standard or for export of coconut-based products.

Table 20.1. Responses to questionnaire (see Appendix 20.1) obtained at Suva meeting. Respondents were Cook Islands, Vanuatu, Papua New Guinea, Solomon Islands, Tonga, New Caledonia, Guam, Fiji and Western Samoa.

Question 1: Importance of coconut	8 say that coconut is extremely important for local use; 5 say that coconut is extremely important for both local use and export; 2 say that coconut is important for export; 2 say that coconut is not important for export; 1 says that coconut is moderately important for local use.
Question 2: Improvement program	7 have a coconut improvement program; 2 feel the need for such a program; 1 feels no need.
Question 3: Varieties used	MD (MRD/MYD), LT (local talls, e.g: Western Samoan Red Tall in WSM; Tongan Tall in TON; Rotuma Tall, Rennell Tall in FJI).
Question 4: Elite trees and special requirements	2 say they have elite trees. Special requirements listed: pest resistance, higher yield, optimisation of land and fertiliser use.

Recent breeding programs have shown the potential for doubling or trebling of yields, especially by hybridising selected dwarf and tall populations. Such yield increases would directly raise the general nutritional standard in the producer countries and could also improve the competitiveness of coconut relative to other oil crops. Among the main goals of breeding are the development of higher-yielding varieties and the control of pests and diseases by resistance.

No international central research organisation exists for coconut, and sustained research on diseases has been limited. Breeding programs continue in the absence of information on intracellular pathogens such as viruses or viroids. Until FAO and ACIAR funded research on viroids and viruses in coconuts, no pathogens of these types had been reported. In particular, the aetiology of cadang-cadang disease (viroid) and coconut foliar decay disease (virus) has been

determined at the Waite Institute in collaboration with colleagues in Asian and Pacific countries.

Previous work at the Waite Institute has led to the development of diagnostic methods, survey and collection protocols, and facilities for molecular characterisation of the coconut viroids. These facilities are now in place and are available for attempts to answer questions relating to the economic importance of the viroids, their interactions with each other (since there is strong evidence that a range of related viroids are present in palms), and their interactions with other pathogens. Of particular concern to breeders, governments and quarantine authorities are questions such as: Why is there no obvious epidemic anywhere outside the Philippines where the related viroids are present? How can the initial sudden outbreak of cadang-cadang in the 1930s be explained, and what is the risk of a similar event being triggered or occurring spontaneously in the areas of the related viroids? How could such viroid-induced diseases be controlled in the Philippines, and also elsewhere, should a new outbreak of a related disease occur? To enable us to answer such questions, information about the relationship of the new viroids to CCCVd is urgently required. Their nucleotide sequences need to be determined so that similarities and differences can be mapped and interpreted.

Analysis of our survey samples has revealed very complex patterns of molecular species of viroids, the interpretation of which is impossible without knowing their nucleotide sequences. It has been found in other host species, such as citrus and grapevine, that multiple viroid infections occur, and that pathogen interactions in these situations are complex. The components of multiple infections can be independent, can help or inhibit each other's replication, or can induce different symptoms alone or in co-infection. CCCVd also exists in progressively different molecular forms depending on the stage of disease. It will be necessary to distinguish between these possibilities when assessing the epidemiological risks of the new viroids and deciding what recommendations to suggest for the exchange of coconut germplasm.

Therefore, objectives for future work would include:

1. Determining the nucleotide sequences of CCCVd-related molecules in order to analyse the exact levels of their relatedness, ascertaining their viroid nature, and assessing the risk of each isolate becoming more virulent for coconuts either spontaneously or by environmental interference.
2. Determining whether the isolates are spread through the germline or infect their host locally, and elucidating their epidemiology.
3. Supplying specific probes, for future screening and surveys, for each particular confirmed viroid of possible economic significance.

A detailed plan for future work is outlined in Figure 20.1 and elaborated below the figure.

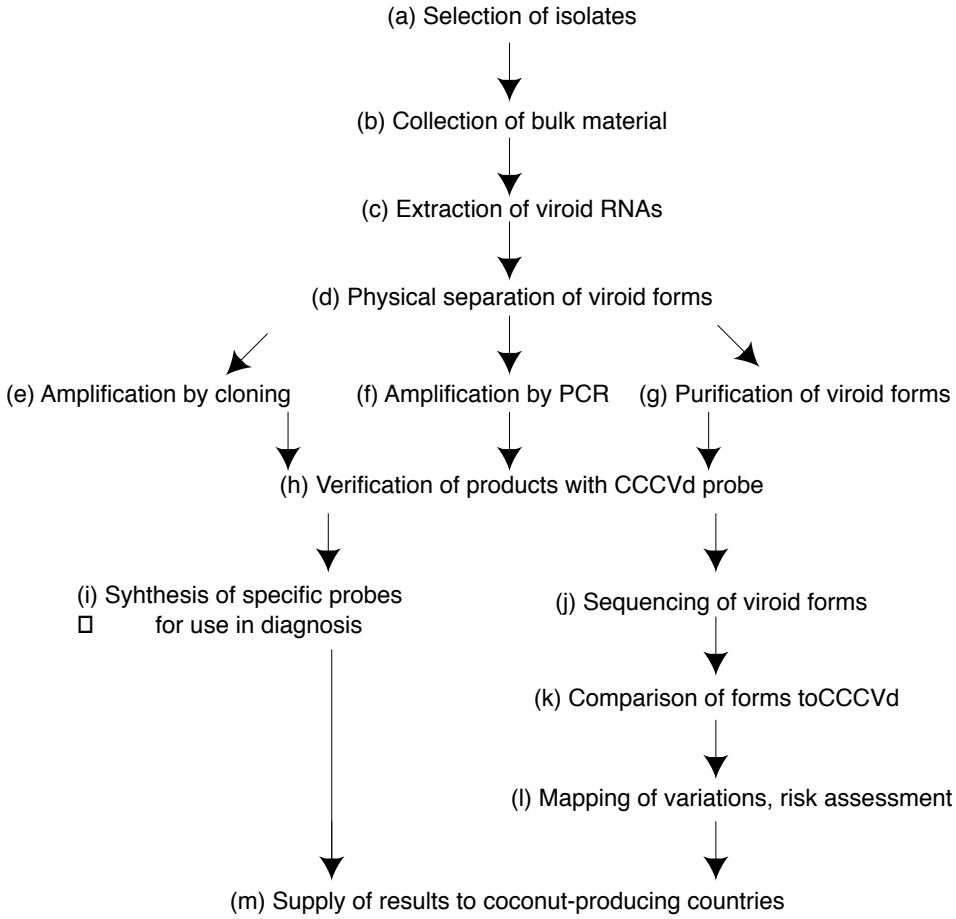

Figure 20.1. Flow diagram for future work.
a, b: Some key isolates will be selected on the basis of our previous survey data; for example:

- **local coconuts from sites with historically high traffic of people and from isolated places, to establish geographical relationships**
- **Malayan Red and Yellow Dwarfs and Rennell Talls from their places of origin and from seed gardens in cadang-cadang-free areas, to determine whether viroid-like nucleic acids are carried through the germline or are locally infected**
- **oil palms with GOS from different areas, to determine whether there are variations in the isolates and whether there is any connection with the local coconut isolates**
- **Pandanus isolates from several places to determine whether Pandanus may be a source, reservoir or alternative host for the coconut isolates. Bulk material will be collected from identified trees in collaboration with local staff and stored at the Waite Institute.**

c, d: Viroid-like RNAs will be extracted using the method established for the survey (Chapter 16), and the different molecular forms present in each sample will be separated as distinct bands by PAGE.
e–h: Amplification of each of the forms will be attempted, either by molecular cloning into a standard cloning vector, or by PCR using primers from the conserved region of CCCVd that are expected to have a high percentage of sequence homology to the conserved regions of the other forms. Alternatively, if sufficiently large quantities of molecular forms can be obtained by extraction of leaves, these could be purified directly from the gel. All products need to have sequences verified by hybridisation assay using CCCVd probe.

i–l: Verified products of e–g will be used to synthesise specific probes as tools for diagnosing the presence of that particular form and distinguishing between forms. Such probes would provide advantages over working with the CCCVd probe, which is only partly homologous to the target sequences, because more stringent assay conditions can be applied and distinction between different isolates will be possible, thus making results more clearly interpretable. The products will also be used for nucleotide sequence determination of the forms. Once the sequences are known, comparison with each other and with CCCVd will elucidate the levels of mutual relatedness. Sequences of the predominant variant will be determined. The main value of this will be to assess the risk of spontaneous or environmentally induced changes toward higher pathogenicity occurring.

m: Results of benefit to coconut producers will be that:

- **specific probes for diagnosis will be available for particular confirmed viroids**
- **risk assessment and recommendations for control can be facilitated.**

Appendix 20.1: Questionnaire for Suva meeting

Name:

Organisation:

Address:

Country:

1. Is coconut an important crop in your country?

for local use	*for export*
( ) extremely important	( ) extremely important
( ) important	( ) important
( ) moderately important	( ) moderately important

2. Do you ( ) have a coconut improvement program?
   ( ) plan to have a coconut improvement program?
   ( ) feel the need for a coconut improvement program?

3. If YES, which varieties or hybrids are/will be the principal parents?

4. Have you identified any elite trees in your country which may be suitable candidates for a breeding program?

21 COCONUT FOLIAR DECAY DISEASE AND IDENTIFICATION OF ITS VIRAL AGENT

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Introduction

Foliar decay is a disease of introduced coconut palm cultivars in Vanuatu that is induced by feeding of the cixiid bug (plant hopper) *Myndus taffini* (Julia 1982). It is also known as 'New Hebrides coconut disease' and 'foliar decay of coconut caused by *M. taffini*'. The disease is economically important because of its present influence on a regional selection and breeding program for coconut palm in Vanuatu.

In the field, gradients of disease occur from the borders adjacent to native forest into the plantation (Fig. 21.1). *M. taffini* (Figs 21.2 and 21.3) breeds on the roots of a tree species (*Hibiscus tiliaceus*) that predominates in the bordering forest (Julia 1982). The presence of this species adjacent to and within plantations (Fig. 21.4) provides foci for the plant hopper.

Figure 21.1. **Aerial view of a variety trial at Saraoutou (Vanuatu Agricultural Research and Training Centre) showing both the gradients of disease incidence from the native forest into the planting and the variation in susceptibility of different varieties. Note particularly the 2 rows of tolerant VTT adjacent to the forest, and the yellow susceptible trees within the block where numerous trees have already died from foliar decay disease. (The dense planting next to the forest is a replanted area.)**

Figure 21.2. **Adult female *Myndus taffini*.**

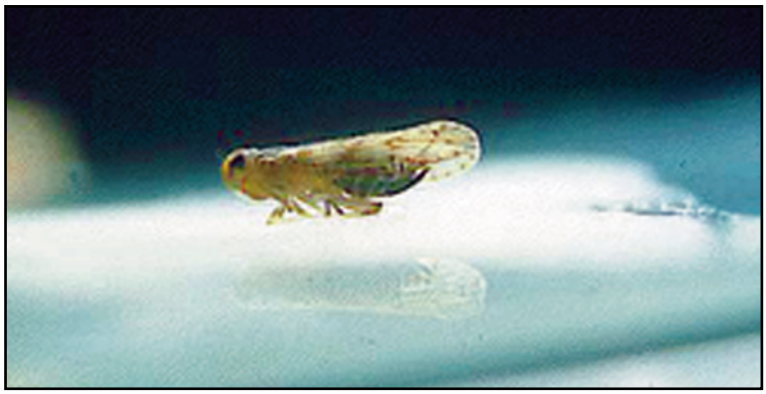


Figure 21.3. **Head (right), and an enlargement of the labium and emergent stylets of an adult *M. taffini***

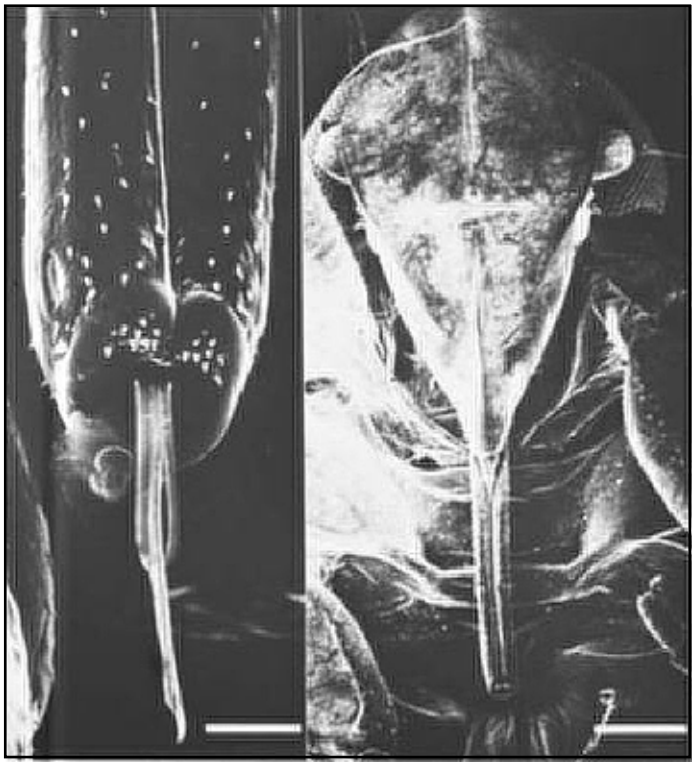


Figure 21.4. **A thicket of *Hibiscus tiliaceus* in a coconut plantation on Espiritu Santo.**

Figure 21.5. **A naturally infected Malayan Red Dwarf palm showing yellowing of fronds in the central whorl at the mid stage of coconut foliar decay disease.**

A range of symptom severities is observed in affected introduced cultivars and their hybrids, whereas the local *Cocos nucifera* L. 'Vanuatu Tall' is symptomless, and its hybrids show mild symptoms (Calvez et al. 1980). The cultivar Malayan Red Dwarf (MRD) is highly susceptible to coconut foliar decay disease (CFD) in the field (Figs 21.5 and 21.6) and is used as an indicator plant for epidemiology and insect transmission studies.

Figure 21.6. A naturally infected Malayan Red Dwarf palm at the late stage of coconut foliar decay disease.

In the field, yellowing first appears in several leaflets of the fronds 7–11 positions down in the crown from the unopened spear leaf. More general yellowing of the fronds ensues and they develop lateral necrosis of the petiole and die prematurely, hanging from the petiole downward through the canopy. Other fronds become yellow and die as they reach the central whorl of the crown. Affected palms characteristically show a normal apex, several yellowish fronds, then several, young dead fronds hanging through green older fronds. The trunk generally narrows and may thicken again if remission occurs, as in a tolerant palm. Susceptible cultivars die between 1 and 2 years after symptoms appear. Spathes at the base of yellow fronds rot if the subtending frond dies, or they produce an inflorescence that develops normally but bears fewer nuts than normal.

Seedlings that are experimentally inoculated in insect-proof cages with *M. taffini* collected in the field show similar symptoms on fronds 3–5 at 6–11 months after inoculation. Collapse of the yellow fronds also seems to coincide with some lateral necrosis at the base of the petiole.

Neither fungi, bacteria nor nematodes have been implicated as the pathogen, and tetracycline applications at rates used to control coconut lethal yellowing have had no effect on disease progress (Julia et al. 1985).

Electron microscopy failed to detect any microorganism or virus in affected tissue, and attempts to detect the coconut cadang-cadang viroid by either gel electrophoretic analysis or molecular hybridisation assay of nucleic acid extracts (J. W. Randles and M. Dollet, unpublished) were negative.

The correlation between the distribution of diseased palms and *M. taffini* in plantations and the demonstration of efficient transmission of CFD by adults of *M. taffini* suggested that the disease is caused by a transmissible agent. It seemed unlikely that an insect toxin was involved because small groups of *M. taffini* can transmit CFD during a short inoculation feed.

This chapter describes the steps that led to the implication of a unique virus of a new taxonomic group as the cause of CFD in Vanuatu.

Identification of a Disease-Associated DNA

Determination of the aetiology of CFD is particularly important so that diagnostic procedures can be developed for application to epidemiology and control methods. An ssDNA of low electrophoretic mobility in 5% polyacrylamide gels was found to be specifically associated with diseased MRD coconut palms (Randles et al. 1986). This disease-associated DNA was extractable in low amounts only. It was further characterised to determine whether it could be used as the basis of a diagnostic assay, and to predict the type of virus particle in which it may be encapsidated. The DNA was spread for examination by electron microscopy; this showed that preparations contain circular molecules of mean molecular weight of approx. 4.3×10^5 daltons. Circularity was also shown by 2-dimensional PAGE and our inability to dephosphorylate and label the molecule by the polynucleotide kinase technique (Randles et al. 1987).

Analysis of the DNA by sedimentation gave an S value of 12S to 15S, which is consistent with its having a molecular weight below that of geminivirus DNA (*s*20, w = 16S at pH 7.0). On denaturing polyacrylamide gels, the DNA has a low electrophoretic mobility relative to its molecular weight; consequently, erroneous estimates of molecular weight can be expected when linear DNA markers are used.

The detection in non-denaturing gels of 2 or 3 bands that have the same electrophoretic mobility in the second denaturing dimension of 2-dimensional gels suggests that, under 'native' conditions, the DNA occurs as 2 or 3 conformers that differ in secondary structure. Variations in secondary structure may explain the broad distribution of DNA in both zonal and isopycnic density gradient centrifugation (Randles et al. 1987).

All previously described plant viruses with ssDNA have been placed in the geminivirus group. Geminiviruses contain either 1 or 2 circular ssDNA molecules of approximately $7–8 \times 10^5$ daltons mol. wt and comprising about 2700 nucleotides (Harrison 1985). The DNA associated with foliar decay is significantly smaller than geminivirus DNA, but is close to the size of the DNA of porcine circovirus (Tischer et al. 1982), which sediments at 12.7S, has a contour length of 633 μm, a length of 1.76 kilobases, and a mol. wt of 5.8×10^5 daltons.

A 20 nm Virus Particle Associated with CFDV DNA

This section describes the isolation of unusual, 20 nm, icosahedral particles that co purify with the CFD-associated DNA and that are considered to be coconut foliar decay virus (CFDV) particles.

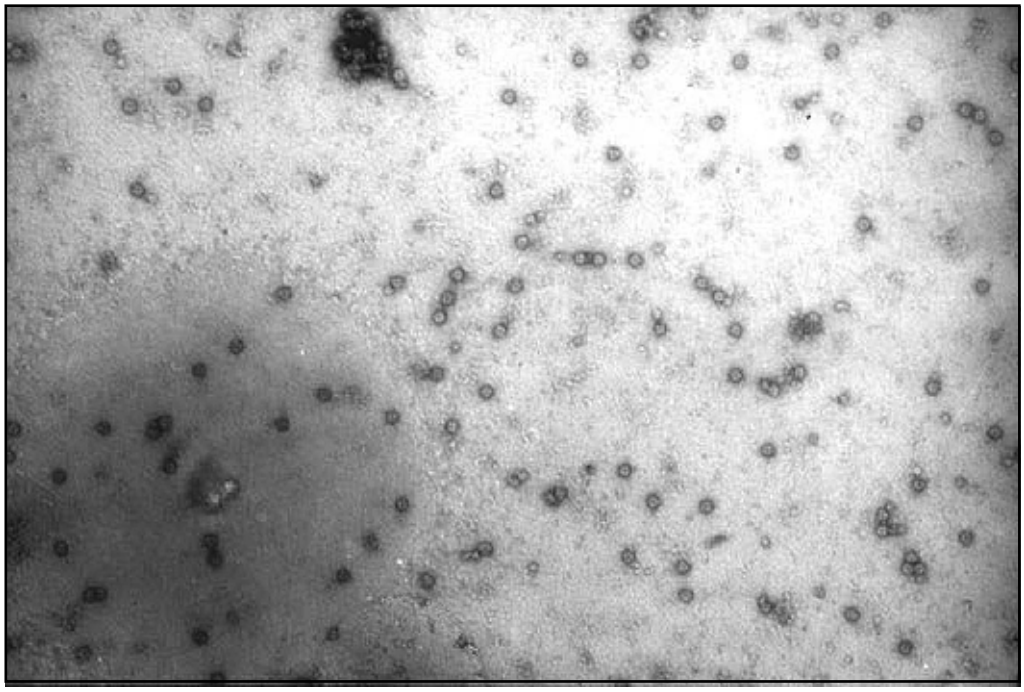
Leaflets from inoculated MRD seedlings (Randles et al. 1986) were chopped and blended, and a component precipitable in polyethylene glycol 6000 was prepared. This was resuspended in 10 mM Tris·HCl (pH 7.2), clarified by centrifugation at 10 000 g for 20 min, and then centrifuged at 360 000 g for 1 h. The pellet was resuspended in 0.6 mL of the Tris buffer and incubated for 1 h at 25° with 0.2 mL of serum from a rabbit immunised against PEG-precipitated material from healthy coconut leaf. The resulting precipitate was removed by sedimentation at 37 000 g for 10 min, and the supernatant was centrifuged at 270 000 g for 1 h. The pellet was resuspended as above and subjected to isopycnic density gradient centrifugation in a 2.4 mL, preformed, 30%–60% Nycodenz (Nyegaard, Oslo) gradient. Centrifugation was done in a Beckman TLA-100.3 rotor at 103 000 g for 15 h at 5°. Fractions (0.2 mL) were displaced from the top of the gradient with Fluorinert FC-40 (Sigma), diluted, and subjected to high-speed centrifugation as above. Pellets were resuspended in the Tris buffer, and aliquots were assayed for DNA by PAGE (Randles et al. 1987) or dot blot hybridisation (Hanold et al. 1988). Electron microscope grids (400 mesh) coated with formvar-carbon films were made hydrophilic by high-voltage glow discharge. Samples were diluted in water, placed on the grids for 5 min and drained, and the grids were washed once with water. They were stained for 10 s with 2% uranyl acetate or shadowed at about 20° with Pt-Pd. Electron microscope magnification was calibrated with a cross-grating replica of 2160 lines/mm (Agar, UK).

Particles of 20.0 ± 0.4 nm (n = 212) in diameter (Fig. 21.7) were exclusively associated with fractions that contained CFD-associated DNA (Nycodenz density range 1.27–1.30 g/mL). The number seen was approximately proportional to the amount of CFD-associated DNA detected in the fraction. Some stained particles appeared hexagonal in outline, and shadowed particles also showed an angular outline, which would be consistent with an icosahedral structure of 12 vertices and 20 faces (T = 1 symmetry; Randles and Hanold 1989). Smaller spherical particles of 12–15 nm in diameter were also detected in lower-density fractions from both healthy and diseased plants, and a few were commonly seen in preparations of the 20 nm particles.

The CFD-associated particles could be extracted from fresh or frozen leaves, and their morphology was not affected by treatment for 1 h at 25°C with chloroform, 50% Cs_2SO_4, 100 mM Tris·HCl (pH 9.0) or 100 mM $MgCl_2$. They swelled in 1% SDS and were disrupted in 100 mM sodium acetate buffer (pH 4.0).

Both CFD-associated DNA and the particles occur in very low amounts in diseased coconut palms, but in the absence of known alternative hosts it was necessary to develop a purification method appropriate to this host. The use of bentonite clarification, absorption with an antiserum specific to healthy coconut palm antigens, and isopycnic Nycodenz gradients was essential for successful partial purification of the particles described here. The co-purification of these particles with the disease-specific DNA, and their exclusive association with diseased palms, allows the conclusion to be made that CFD is a virus disease and that the particles described are those of coconut foliar decay virus (CFDV). Hereafter, the disease-associated DNA is termed CFDV DNA.

Figure 21.7. **Electron micrograph showing spherical particles of purified CFD virus, negatively stained with uranyl acetate. Particle diameter is 20 nm.**



The infectivity of the particles has yet to be tested, but their detection will allow the development of serological tests for determining the epidemiology of CFD. The particles are similar in size and symmetry to half-geminate (Hatta and Francki 1979) particles and to subterranean clover stunt virus (SCSV) particles (Chu and Helms 1987). They differ from these other plant viruses in that their single-stranded circular DNA is intermediate in size between that of geminiviruses (ca 2700 nucleotides) and SCSV (900–1000 nucleotides). They most closely resemble the porcine circovirus (Tischer et al. 1982).

Diagnosis of CFDV Infection Using DNA Detection Methods

PAGE

CFDV infection can be recognised in coconut by gel electrophoresis. CFDV DNA migrates as a single band in denaturing polyacrylamide gels, but generally as 2 bands in non-denaturing gels (Randles et al. 1987). Routine diagnosis by this method lacks proof that bands have nucleotide sequence similarity to CFDV. Therefore, hybridisation assays for the DNA should be done in conjunction with PAGE.

Hybridisation with partial sequences

Cloning of CFDV DNA was attempted at first to determine whether its nucleotide sequence was represented in either the DNA of the host plant or the DNA of a geminivirus infecting the grass *Digitaria sanguinalis* in the plantation understorey. The sequences in the cloned fragments were uniquely associated with CFD-infected but not healthy palms and showed no homology with the

D. sanguinalis geminivirus DNA. CFDV is, therefore, unique; this supports the conclusion that CFDV is a member of a previously undescribed taxonomic group.

The availability of cloned partial sequences allowed the development of specific and sensitive molecular hybridisation tests for identifying and characterising CFDV DNA (Hanold et al. 1988) The hybridisation tests were more sensitive than PAGE assays for coconut palms, and probably have broader applicability to other species for which the PAGE nucleic acid preparation procedure developed for coconut palms may not be appropriate. Variation in the strength of the signal is presumably due to differences in CFDV DNA concentration in tissue, sample preparation and assay procedures. Palms assayed within about 8 months of inoculation, just after the first appearance of symptoms, generally had higher concentrations of CFDV DNA than palms infected by natural exposure in the field. Unreliability of diagnosis for CFD by symptoms was expected because of the variation in responses by different palm cultivars (Calvez et al. 1980). Only MRD palms show characteristic symptoms of the disease.

Detection of CFDV DNA independently of symptomatology allows a more reliable evaluation of epidemiology to be done. For example, hybridisation assays indicate that the symptomless VTT coconut palm variety can be infected, and raise the possibility that this variety, which is the main component of plantations in Vanuatu, could act as a latent reservoir of virus infection for sensitive varieties. Neither *H. tiliaceus*, the host of *M. taffini*, nor geminivirus-infected *D. sanguinalis* contained detectable sequences of CFDV DNA, and neither therefore could be implicated as a virus source. Further development of more reliable and faster dot blot assays will allow other species to be assayed on a large scale as potential natural hosts of CFDV DNA. The lack of homology between the *D. sanguinalis* geminivirus DNA and CFDV DNA agrees with the observation that the 2 disease agents have different vectors (*Nesoclutha declivata* and *M. taffini*, respectively). Further development of the assay is described in the next section.

Hybridisation with Near-Full-Length Cloned cDNA and DIG cRNA

The assay for CFDV was greatly improved by using a 1203 bp DNA, amplified by PCR from circular, single-stranded, 1291-nucleotide CFDV DNA (Rohde et al. 1990; Randles et al. 1992). This was used as a template for the synthesis of ^{32}P-labelled cDNA probes. The higher specificity and sensitivity of the assay allowed CFDV DNA to be detected reliably, despite its low concentration in coconut tissue. This hybridisation technique was then used to locate CFDV DNA in various tissues of palms and indicate the level of sampling needed for reliable diagnosis; to show the vascular location of CFDV in rachis and leaf; to show that CFDV DNA can be detected in the body of the vector; and to show that, in cases where the signal was sufficiently discrete, it was over the abdomen rather than over the head.

A non-radioactive probing system was also developed as an alternative detection method. This used digoxygenin-(DIG) labelled complementary RNA (cRNA) (Randles et al. 1992). Generally, all steps in diagnostic tests must be optimised. It was necessary to inhibit tannin production during extraction of leaf and to include an alkali incubation step. Tests with the non-radioactive DIG cRNA probe using antibody against the probe and an alkaline-phosphatase-based assay required a more rigorous alkali incubation than tests with the radioactive probe. This was necessary to prevent development of a non-specific endogenous reaction with the non radioactive probing system. We have previously observed apparent endogenous phosphatase activity in coconut nucleic acid extracts (D. Hanold and J. W. Randles, unpublished) that was highly stable and interfered with colorimetric detection methods.

Applications of the hybridisation assay

CFDV DNA was distributed unevenly in whole plants and in an apparently non-systematic pattern (Randles et al. 1992). Thus, multiple sampling is needed for reliable diagnosis. The result of the examination of 29 whole palms indicated that root sampling should be combined with leaf sampling, particularly where new areas are being surveyed for CFDV incidence. We conclude that no preferred sampling position on leaflets or on fronds is indicated, but a minimum sampling should include tissue from fronds 1, 3 and 5 and secondary roots.

To localise virus-containing tissue, hybridisation and macro- and micro-autoradiography were done with unsupported intact sections of coconut rachis. This was possible because of the structural strength of this tissue. Mounting of tissue was necessary only for the application of gelatin and photographic emulsion. The location of the virus as indicated by the probe, in vascular bundles and in phloem in particular, probably accounts for the low recovery of virus from tissue and the need to use alkali maceration to release CFDV DNA for hybridisation assay.

Symptoms of CFDV infection in highly susceptible cultivars are yellowing of leaflets in upper to central fronds and the progressive necrosis of the outer edge of the petiole. This necrosis is the site of collapse, causing the death of fronds. Autoradiography of a diseased palm showed that the virus DNA was present in a young unaffected frond, and both within and adjacent to the necrotic region at the early stages of lesion development. It was not detected within the necrotic zone of an older frond of the same tree, but it was detected in the non-necrotic zones either side of this older lesion. These observations would be consistent with CFDV being associated with and causing this necrosis, then degrading several months after the development of necrosis. No reason is known for the localisation of necrosis on petioles in a particular part of the crown. The only other symptom, namely yellowing of leaflets, may be associated with cytopathic effects in phloem and reduced carbohydrate translocation.

CFDV can be detected in leaves of seedlings within 6 months of controlled inoculation with *Myndus*, and symptoms appear 1–4 months later. Palms infected by natural exposure in the field show CFDV in most tissues sampled within 4–7 months of symptoms first appearing. The disease-free trees, including tolerant cultivars, and palms in disease remission generally contain CFDV DNA in leaflets and rachis; a large reservoir of virus can therefore exist in apparently disease-free coconut-growing areas.

Although CFDV DNA was detected in embryos, no transmission of CFD through seed has been shown. No CFDV DNA was detectable in pollen samples. However, husks contain CFDV, and movement of nuts can allow transfer of virus to new sites. It will be necessary to determine whether vectors can acquire virus from green husk to decide whether there are quarantine risks from the movement of nuts.

The vector, *M. taffini*, commonly settles on coconut fronds at the junction of leaflet with rachis, where our in-situ studies have shown that virus is likely to be available. Observations of feeding sites and behaviour should now be attempted to determine whether virus may be acquired by phloem feeding. The frequent detection of virus in coconut roots raises an alternative possibility that the soil-inhabiting larvae of *Myndus* could acquire virus from or infect coconut roots. We have not detected CFDV in the dicotyledon host of *Myndus* larvae, *H. tiliaceus*, but the frequently occurring proximity of coconuts to *Hibiscus* could allow acquisition of virus by larvae from roots of infected coconut palms growing adjacent to roots of *Hibiscus*. The association of CFDV DNA with the abdominal areas of *Myndus* is consistent with accumulation

of virus in the gut. Experiments now need to be done to determine whether the virus accumulates or replicates in the insect or its eggs, and whether it has a semi persistent, circulative or propagative association with its vector.

The Nucleotide Sequence of CFDV DNA

To obtain sequence data on CFDV DNA, a single-stranded (ss), circular, covalently closed (ccc) DNA associated with coconut foliar decay virus (CFDV) was purified, amplified by the polymerase chain reaction and subcloned. Its sequence was established by analysis of overlapping, subgenomic cDNA clones (Rohde et al. 1990). The complete sequence comprised 1291 nucleotides and contained open reading frames for 6 proteins of molecular weight larger than 5 kDa (Fig. 21.8). One of these (ORF1, 33.4 kDa) codes for a leucine-rich protein with the nucleoside triphosphate-binding motif GXGKS (glycine-x-glycine-lysine-serine), and may possibly participate in virus replication. The putative viral protein encoded by ORF3 (6.4 kDa) is a positively charged, arginine-rich protein with homology to the capsid protein of nuclear polyhedrosis virus, and may represent the CFDV coat protein. CFDV DNA can form a stable stem structure of 10 GC base pairs subtending a loop sequence that, in one orientation, closely resembles the motif TAATATTAC, conserved in a similar structural arrangement within the geminivirus group (Fig. 21.9). Otherwise, no sequence homology to DNA-containing plant viruses of the gemini- or caulimovirus groups was found (Rohde et al. 1990). CFDV therefore represents a new taxonomic group of plant viruses.

Figure 21.8. **Nucleotide sequence of the CFDV DNA. Open reading frames (ORFs) are presented only for the (+) orientation of the DNA in the virus and are given in the 1 letter amino acid code. Shaded area represents the NTP-binding motif glycine-x-glycine lysine-serine. Arrows show position of the inverted repeat sequence capable of forming a stable stem-loop structure (Fig. 21.9).**

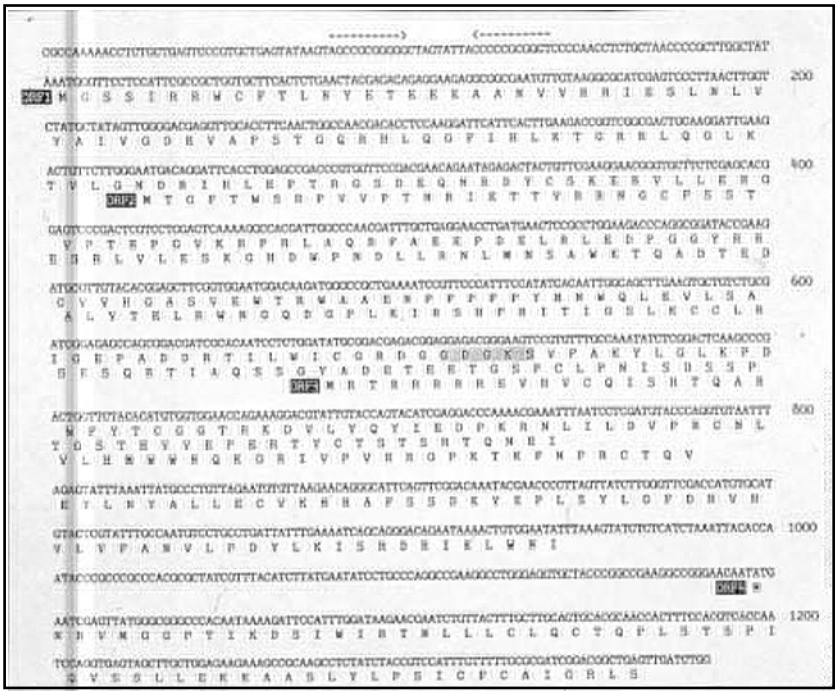
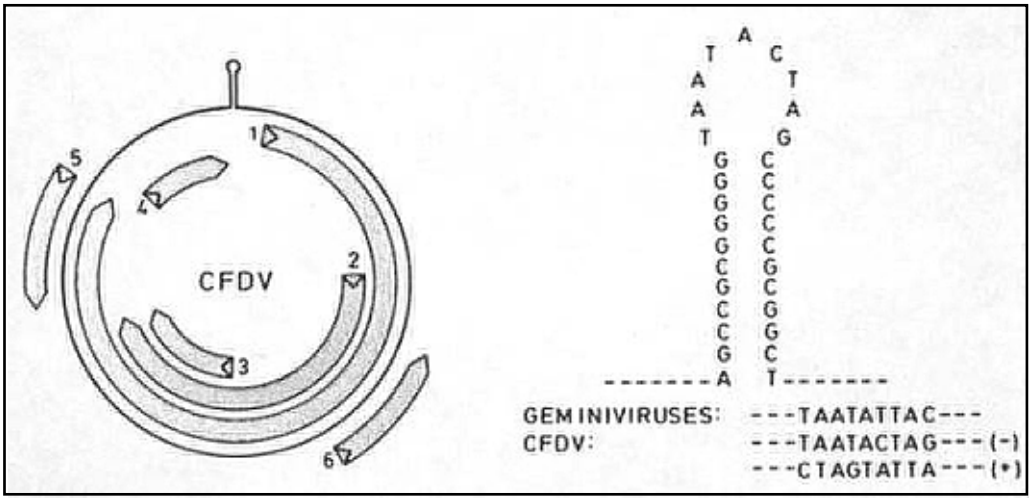


Figure 21.9. **Organisation of the CFDV DNA. Left: Putative ORFs (numbered shaded areas) and the relative location of the potential stable stem-loop structure. Right: The stem-loop structure is represented for the (–) orientation. Loop sequences are given for both CFDV orientations and compared with the canonical geminivirus motif.**



22 A POTYVIRUS ISOLATED FROM *ROYSTONEA REGIA* PALM

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Introduction

Palms are widely grown throughout the tropics and subtropics as either food and fibre crops or as ornamentals, but surprisingly few virus diseases have been described. Rod-shaped particles have been detected in coconut palms (*Cocos nucifera)* from the Philippines and Malaysia, but they were not typical of virus particles and were not specifically associated with disease (Randles 1975a). A potyvirus is associated with palm mosaic disease of *Washingtonia robusta* palms in California (Mayhew and Tidwell 1978). Coconut foliar decay virus has 18 nm diameter isometric particles, an ssDNA genome and a cixiid bug vector and occurs on coconut palms in Vanuatu (Randles and Hanold 1989). None of these viruses has been reported from Australia.

This chapter reports the isolation and some characteristics of a rod-shaped virus from ornamental Cuban royal palms (*Roystonea regia*) in Queensland with chlorotic ringspot symptoms.

Methods

Virus isolates and transmission studies

Virus isolates (*Roystonea* palm virus, RPV) were obtained from naturally infected *Roystonea regia* palms with chlorotic ringspot symptoms at Brooloo (isolate 321) and Gumdale (isolate 349) in Queensland. All studies were done with isolate 321 except where noted. For transmission tests, extracts were prepared in 0.1 M potassium phosphate (pH 7.0) including 0.1% Na_2SO_3, or in tap water, and inoculated onto the leaves of carborundum-dusted test plants. All plants were maintained in an insect-proof glasshouse and were regularly sprayed with insecticides.

Colonies of the aphid *Myzus persicae* were maintained on radish (*Raphanus sativus*). For transmission studies the aphids were allowed an acquisition access period of 15–60 s on RPV-infected *Nicotiana benthamiana*, placed on test plants (5 aphids per plant) for an overnight inoculation access period, and then sprayed with an insecticide.

Electron microscopy

Field samples of *R. regia* infected with RPV were fixed and embedded for electron microscopy as described by Greber and Gowanlock (1979).

For negative staining of sap preparations, extracts were placed on nitrocellulose-coated, carbon-stabilised grids and contrasted with 1% (w/v) ammonium molybdate (pH 5.8) or 2% (w/v) potassium phosphotungstate (pH 7). To determine particle length, virions from partially purified extracts of RPV-infected *Nicotiana clevelandii* (M. Pearson and J. W. Randles, unpublished) were measured using the IMAGE 1.41 program and catalase crystals as a size standard.

Serology

The presence of potyvirus particles in *N. benthamiana* infected with isolate 349 and *C. quinoa* infected with isolate 321 was confirmed by the use of the *Agdia potyvirus* group test (Agdia Inc, Elkhart, Indiana). The test was conducted according to the manufacturer's instructions, and samples were considered positive when the $A_{405\ nm}$ value was greater than the mean plus 3 standard deviations of appropriate uninfected controls.

Results

Symptoms, transmission, and experimental host range

Naturally infected *R. regia* palms displayed chlorotic ringspots on the fronds (Figs 22.1a, b). New growth on infected palms was stunted, and the plants lacked vigour and eventually died prematurely. RPV was mechanically transmitted directly from *R. regia* palms to *Chenopodium quinoa* (isolate 349 only), *C. amaranticolor* and *N. benthamiana*.

Isolate 321 caused the following symptoms in mechanically inoculated test plants:

- *C. amaranticolor*—minute, chlorotic local lesions after 5 days, developing necrotic centres and red borders, followed by systemic chlorotic flecks and epinasty (Fig. 22.2)
- *C. quinoa*—1–2 mm chlorotic local lesions in 5 days, developing necrotic centres and coalescing, followed by systemic chlorotic flecks and epinasty (Fig. 22.3)
- *Cucumis sativus* cv. 'Green Gem'—necrotic local lesions after 12 days
- *N. benthamiana*—systemic vein clearing and mottle after 6 days
- *N. clevelandii*—systemic, diffuse, 1–3 mm chlorotic spots after 9 days
- *N. tabacum* cv. 'Xanthi n.c.'—diffuse, chlorotic local lesions after 10 days, no systemic infection.

Only some inoculated plants of *Vicia faba* cvv. 'Aquadulce' and 'Early Long Pod' became infected. Chocolate-coloured target spots appeared on the older leaves of some cv. 'Aquadulce' plants 20–25 days after inoculation, followed by systemic, diffuse chlorotic spotting on younger shoots. Some plants of cv. 'Early Long Pod' developed systemic necrotic spots, or mosaic, or both, on the leaves and stems. Infection in these hosts was confirmed by electron microscopy of sap or by back-inoculation to *C. quinoa*. Isolates 321 and 349 caused similar symptoms on *C. amaranticolor*, *C. quinoa* and *N. benthamiana*.

No symptoms were observed, and no systemic virus infection was detected by back inoculation to *C. quinoa* in the following mechanically inoculated test plants: *Datura stramonium*, *Phaseolus vulgaris* cv. 'Bountiful', *Pisum sativum* cv. 'Greenfeast', *Glycine max* cv. 'Bragg', *Nicotiana glutinosa*, *Sorghum bicolor* cv. 'White Trojan', *Vigna unguiculata* cv. 'Blackeye', *Zea mays* cv. 'Supagold'. Aphid-inoculated plants of *Washingtonia robusta* did not develop any symptoms and the virus was not detected in them by back-inoculation to *C. quinoa*.

Figure 22.1. **Chlorotic ringspot symptoms on the fronds of *Roystonea regia* palm infected with RPV.**
Figure 22.1a. **Distal part of frond.**

Figure 22.1b. **Isolated leaflet.**

Figure 22.2. **RPV-infected *Chenopodium quinoa* leaf showing chlorotic local lesions (left, centre), and uninfected leaf (right).**

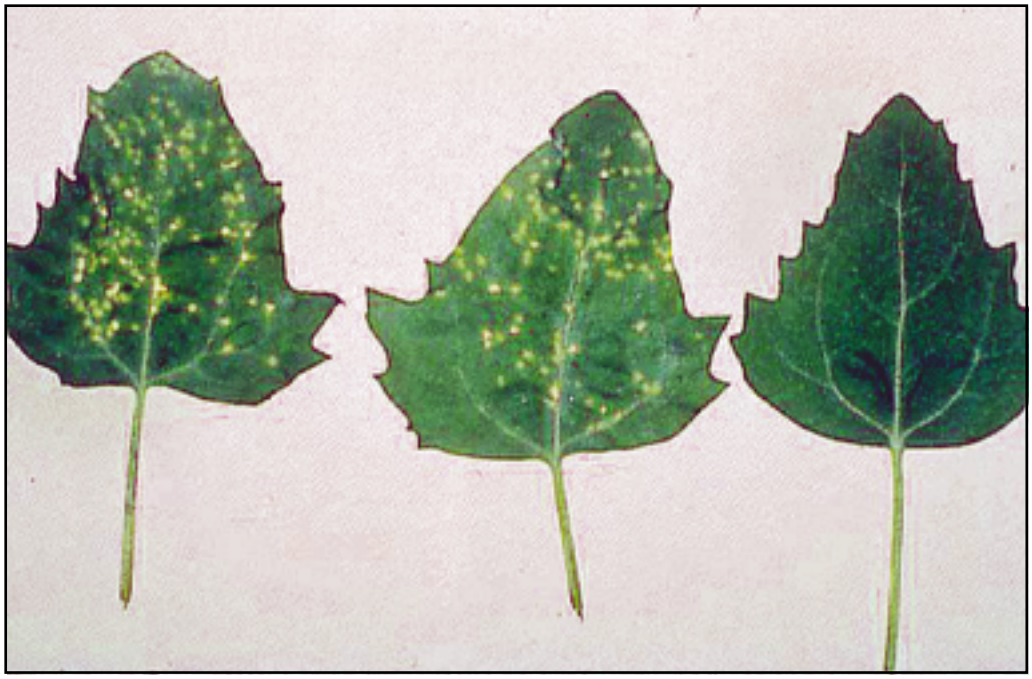
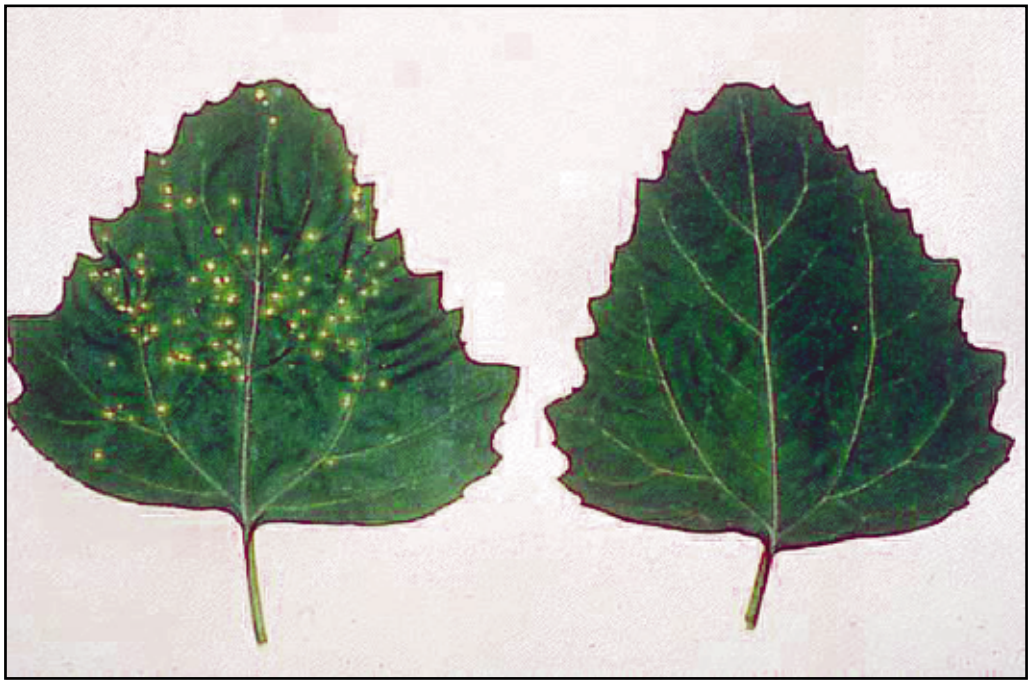


Figure 22.3. **RPV-infected *Chenopodium amaranticolor* leaf showing necrotic local lesions (left), and uninfected leaf (right).**

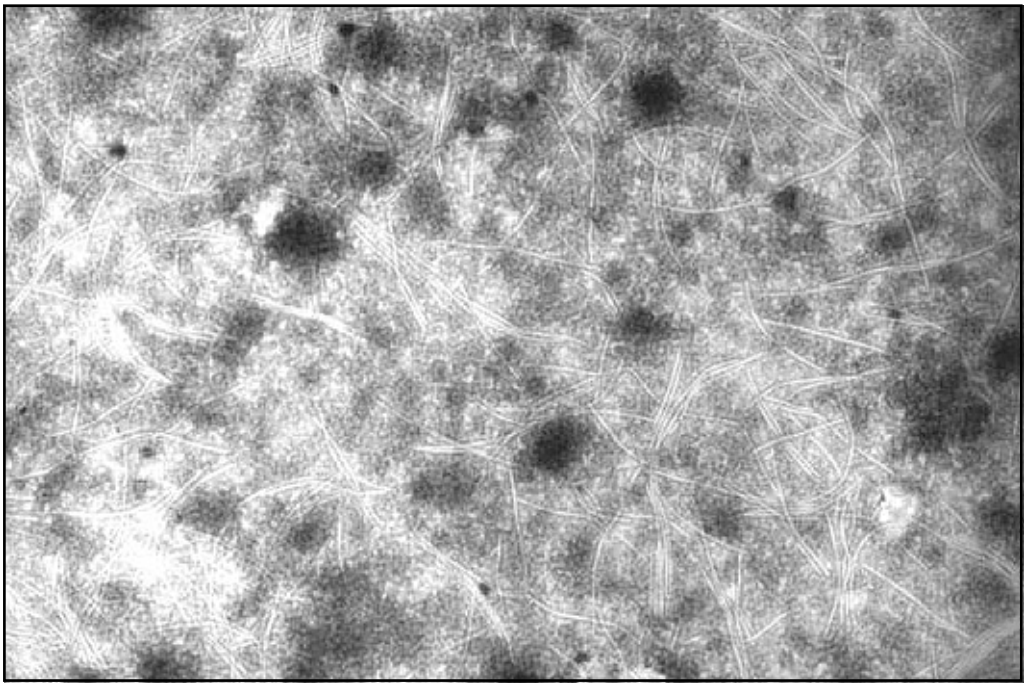


RPV was transmitted by *M. persicae* to 3 out of 6 *N. benthamiana* seedlings, in which typical disease symptoms developed and in which virus particles were detected by electron microscopy of sap.

Electron microscopy

The virions of RPV were flexous rods, 742 nm ± 21 nm long (mean of 90 particles from the 2 largest size classes from a total of 100 particles measured; Fig. 22.4). Infected cells of *R. regia* contained pinwheel inclusions and laminated aggregates typical of potyvirus infections (Fig. 22.5).

Figure 22.4. Particles of RPV, negatively stained with 1% (w/v) ammonium molybdate, pH 5.8.



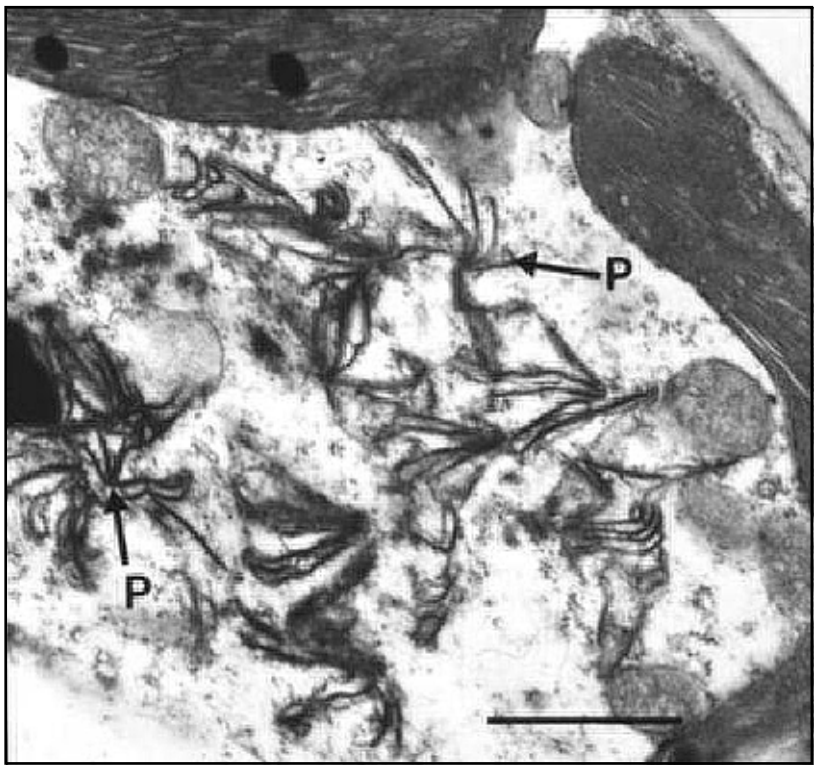
Serology

In indirect ELISA with the Agdia monoclonal antibody for the potyvirus group, the $A_{405\ nm}$ values for uninfected and isolate-349-infected *N. benthamiana* were respectively 0.01 and 0.23, and for uninfected and isolate-231-infected *C. quinoa* were respectively 0.02 and 0.46.

Discussion

The characteristics determined for RPV were consistent with classification in the potyvirus group. Virus particles were flexous rods with a mean length of 742 nm and reacted with the potyvirus group antibodies in ELISA. The virus was aphid-transmitted, and infected leaf cells contained pinwheel inclusions typical of the potyvirus group.

Figure 22.5. Pinwheel inclusions in the cells of RPV-infected *Roystonea regia* palm. Bar represents 1 μm.



The symptoms produced by RPV in *R. regia* were similar to those of 'palm mosaic' caused by potyvirus infection of *W. robusta* palms in California (Mayhew and Tidwell 1978). However, the 'palm mosaic' virus was not mechanically transmitted to a range of herbaceous indicators, including *C. amaranticolor* and *C. quinoa*, which are susceptible to RPV, and we failed to transmit RPV to *W. robusta*. The 'palm mosaic' virus was not reported to occur in or to be transmitted to any palm species other than *W. robusta*. An antiserum to the 'palm mosaic' virus was not produced, and preserved virus-infected tissue is no longer available (D. E. Mayhew, pers. comm.). This precludes further comparison between these 2 viruses.

Further studies are under way to determine whether RPV is a distinct, previously undescribed potyvirus, though it may not be possible to determine whether RPV and the 'palm mosaic' virus are synonymous.

Acknowledgments

We thank Don Gowanlock for assistance with electron microscopy and Keith Bodman for the supply of palm seedlings for transmission studies.

23 SOCORRO WILT DISEASE OF COCONUT PALM

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Two visits were made to Socorro, Mindoro Island, to investigate the Socorro wilt disease. The first was to isolate nucleic acids for hybridisation assay with CCCVd-specific probes. The second was to describe the disease and to suggest research that may help in identifying the cause.

Visit I: March 1990, D. Hanold

Socorro wilt disease (SWD) was first reported on Mindoro Island, Philippines, around 1960, and has been investigated since 1977. During this period approximately 1000 new infections have been observed per year. Some areas are so badly affected that coconut production has been abandoned. The disease seems to 'jump' over long distances, often causing new foci of infection several kilometres away from previous centres of disease.

Samples were collected in the Socorro area from 6 symptomatic (1 with early, 2 with medium, 3 with late-stage SWD symptoms) and 6 asymptomatic coconut palms, and from 2 herbaceous monocotyledonous plants in the Zingiberaceae growing near SWD palms. Assays for CCCVd-related nucleic acids and analysis of results were done as described in Chapter 16.

The results are presented in Table 23.1. They show that the samples from trees classified as having medium- or late-stage SWD symptoms are the only ones with bands in the viroid region binding CCCVd-probe at high stringency, indicating the presence of viroid-like sequences closely related to CCCVd in these palms. However, whether this finding actually means that the wilt is caused by a CCCVd-related viroid, or whether other explanations are appropriate (for example, secondary infection of SWD-affected palms by viroid-like nucleic acids), will need further investigation.

Table 23.1. Results of analysis of Socorro samples.

Host	Symptoms	Number sampled	Percentage positive with CCCVd probe*			
			Overall		Viroid region	
			LS	HS	LS	HS
Coconut	None	6	66	50	33	0
Coconut	Early-stage SWD	1	100	100	0	0
Coconut	Medium-stage SWD	2	50	50	50	50
Coconut	Late-stage SWD	3	100	100	100	33
Zingiberaceae	None	2	100	100	50	0

* LS = low-stringency, post-hybridisation wash; HS = high-stringency, post-hybridisation wash (see Chapter 16 for details).

Visit II: July 1991, J. W. Randles

Socorro wilt disease (SWD) (Abad 1981; Abad et al. 1980) is a disease of unknown aetiology recognised only in the barangays adjacent to Socorro, Oriental Mindoro, Philippines. It was first reported to the Philippine Coconut Authority (PCA) from an apparent primary focus near Pola in 1977 and has spread in a series of jumps to a radius of about 13 km. Each of the secondary foci appear to be small and discrete; a series of epidemiological surveys and analyses have provided a comprehensive set of data describing the origin, distribution and spread in relation to time, and occurrence in relation to topography, soil and associated plant species (Abad 1981; Concibido et al. 1984; San Juan 1984; San Juan et al. 1980, 1986).

The effects of the disease have been to kill more than 12 000 palms, to induce many growers to abandon coconut growing, often in favour of citrus, and to present a threat to coconut production elsewhere in the Philippines. Diseases of similar pathology have been reported from Tanzania (Steiner et al. 1977) and Natuna Island, Indonesia (Sitepu 1983), but the causes remain unknown or unproven. Lethal Yellowing causes death of palms at a similar rate to that of SWD, but is treatable by tetracycline antibiotics and differs in pathology from SWD. An attempt to suppress SWD with tetracycline was negative, although only low doses were tested.

No control measures are known, and the aetiology of the disease (Abad et al. 1982; Concibido et al. 1984) needs to be determined to allow the development of control procedures, to evaluate the risk of spread of SWD to other areas, and to map the distribution of a presumed agent in relation to distribution of disease.

Review of SWD

Epidemiology

- A lethal wilt of coconut in Socorro was first reported in the early 1960s.
- PCA notified of SWD in 3 barangays in 1976.
- Affected palms are less than 25 years old.
- SWD spread described as a jump-spread pattern.
- SWD could not be controlled by eradication campaigns in 1977, 1978 and 1980.
- Mean incidence in the 5 years 1977–81 was in the range 0.9%–4.1%.
- SWD occurs preferentially at 110–190 m above sea level.
- Highest incidence is in 11–15-year-old palms, then progressively lower at 16–20 y and 21–25 y.
- SWD incidence is virtually nil in palms aged 1–5 y and 26–35 y.
- *Oryctes* (rhinoceros beetle) damage is higher in SWD-prevalent areas.
- *Imperata cylindrica* and *Mikania cordata* incidence was positively correlated with the incidence of SWD.
- *Paspalum conjugatum* incidence was negatively correlated with the incidence of SWD.
- Soil cation (Ca^{++} and Mg^{++}) exchange capacity and percentages of sand and silt are positively correlated with SWD incidence; percentage of clay and pH are negatively correlated.
- SWD has spread to a range of 13 km from its presumed focus in the early 1960s.
- Distribution fits 2 models as follows: $y = 0.49; x^{-1.25}$ and $y = 0.35; xe^{-0.33x}$ (where y = incidence and x = distance).

- The isolation distance required to reduce disease incidence to 5% is approximately 6.2 km.
- Increase in disease incidence seems to be best explained by a simple interest model; this implies a monocyclic system.
- The pattern of disease follows a negative exponential curve, with rapid growth early and slow growth later.
- Apparent infection rates give a doubling time for SWD increase of about 13 years.

Development of disease

- The earliest observed symptom is premature senescence of the oldest fronds, then proceeding upwards into the crown.
- Distal browning of leaflets is followed by browning and desiccation of all leaflets on the frond; this progresses to the next-younger fronds.
- Affected fronds hang from a buckled rachis, often broken midway.
- Premature nutfall occurs, inflorescence production stops, unopened primordial spathes rot, roots blacken and rot.
- Nuts forming during disease development are small and oblong, frequently with a dry kernel.
- The top ca. 1 m of the trunk is corrugated vertically, possibly indicating a shrivelling owing to lack of turgor.
- Internal tissue in the top ca. 1 m of the trunk shows a central vertical split with light brown discolouration of fibre bundles.
- At the latest stages (when the crown is reduced to 4–6 surviving fronds), rotting of an external layer about 1 cm thick affects about a third of the trunk base.
- Death of palms occurs 3–7 months from the appearance of first symptoms.

Determination of aetiology

Negative results have been reported by R. Abad and N. San Juan for pathogenicity tests with the following organisms, which were found near or cultured from SWD-affected trees. Tests were evaluated 12 months after inoculation.

- 2 cultures of bacteria inoculated at the base of seedlings
- 1 unidentified Phycomycete fungus inoculated at leaf and stem base
- 1 unidentified Basidiomycete fungus at leaf and stem base
- Nematode *Xiphinema americanum* inoculated to roots
- Nematode *X. insigne* inoculated to roots
- Nematode *Helicotylenchus* sp. inoculated to roots
- Nematode *Rotylenchus* sp. inoculated to roots
- Nematode *Meloidogyne* sp. inoculated to roots.

Negative results were also reported using as inocula:

- sap extracts from diseased tissue in tap water, inoculated by pin pricking and mechanical rubbing
- blended diseased roots added to soil around test seedlings
- rhizosphere soil of diseased palms, used as planting medium for test seedlings.

Standard tests for coconut cadang-cadang viroid at Albay Research Center showed no viroid associated with diseased palms. A test using molecular hybridisation for detection of viroid (D.

Hanold, see above) showed that the diseased palms contained cadang-cadang-related sequences, but further studies would be required to evaluate whether their presence is causally correlated with SWD.

Electron microscopy showed no virus-like, mycoplasma-like or rickettsia-like particles in tissue of the leaf and rachis of frond 14. Current studies involve the planting of seedlings adjacent to infected trees to determine whether disease is transferred preferentially to these. Further isolations of root-associated bacteria and fungi are proceeding for new pathogenicity tests. One palm is reported to have shown a temporary remission of symptoms following 2 injections of 200 mg oxytetracycline at an interval of 4 months. Further injection experiments are suggested to follow up this result (see recommendations).

Tissue pathology

The leaflets with yellowing and browning have been reported to show no specific microscopic changes. The parenchyma cells of the rachis of diseased fronds are generally reduced in size, and phloem cells appear shrunken (Abad 1981).

Biological changes

A study in progress by Dr E. M. T. Mendoza at UPLB has noted increases in specific coumarins in the meristematic zone of the crown of medium- and late-stage SWD palms, but not in early-stage palms (pers. comm; full report to be submitted by Dr Mendoza). Other changes were most evident in the late-stage palms.

Time course of disease-associated changes in the palm

Table 23.2 is a synthesis of published and unpublished information from colleagues, and shows the presumed sequence of events leading to death of SWD-affected palms. The changes in inflorescences are not included here (see previous sections).

No accurate time course has been described, but all observations reinforce the view that primary changes occur in the frond at the base of the crown; these are distinguished from normal senescence by the distribution of dry brown leaflets at the distal end of the frond and green leaflets at the proximal end. The more spectacular symptoms in the trunk and roots occur later.

Table 23.2. Changes associated with Socorro wilt in coconut palm.

Stage	Leaflets	Fronds affected	Upper trunk	Lower trunk	Roots
Early	Distal become brown	2 oldest	No change	No change	No change
Medium	All brown	> 6 oldest	Shrinkage 0.5–1. 5 m from crown, central split to 1 m	Basal peripheral browning; wet rot, odourless	Brown to black
Late	All brown	All except youngest	Vascular browning in bundles below crown	As above	As above

Main Findings and Conclusions

Rationale to the investigation

The attempt at Davao Research Centre (DRC) to implicate non-obligate cellular pathogens such as bacteria, nematodes or fungi as the agents of SWD has been comprehensive but unsuccessful. The possibility that endogenous pathogens such as mycoplasmas, viruses or viroids, or obligate bacteria or fungi, could be involved has not been excluded by previous work. The difficulty of pathological work with coconut palms is a major hindrance to such studies.

The approach adopted here was to try to identify the primary changes associated with the early-stage disease, as those described later in the disease (Table 23.2) could all be considered to be part of a 'cascade' of events derived from the primary changes.

Examination of rachis of early-stage palms

An example of symptoms in the early stage of the disease is shown in Figure 23.1, in which basal fronds become yellow and then brown. The interior of the rachis of these fronds is readily observed by splitting fronds longitudinally from the distal end. As shown in Table 23.3, the rachis of browning fronds (but not of young green fronds) showed internal necrotic zones proximal to the region of leaflet browning (Figs 23.2, 23.3, 23.4, 23.5). The internal lesions differed from those associated with exterior, lesion-producing fungi (Fig. 23.6). Below their apparent origin (Fig. 23.4), a spreading, light- to dark-brown lesion developed, which did not exteriorise on the rachis and which was essentially undetectable from the outside of the rachis. Sequential shaving of the lesions indicated that they spread downward and laterally, and that they were dry and brown with a pale 'water-soaked' zone surrounding the brown zone (Fig. 23.5). The lesions produced a zone of weakness, and some fronds had broken at the site of a lesion in a manner characteristic of the disease.

The rachis and frond of mid-stage (Fig. 23.7) and late-stage palms showed no such internal lesions, but did show a general, pale-brown discolouration of the rachis fibres.

In one example of a palm at the medium stage, the base of the lowest affected frond showed a dark lesion (Fig. 23.8) that was connected to a necrotic zone on the trunk (Fig. 23.9). The base of the frond above did not show this type of lesion (Fig. 23.10). Sectioning of the trunk in this region showed that many vascular bundles had become brown (Figs 23.11, 23.12). Figure 23.13 shows the basal lesion on the trunk, which seems to be characteristic of the late stage.

At one other site (Mabuhay) where early-stage disease symptoms were not typical, lesions were not clearly identified. However, there is a strong correlation between the incidence of these lesions and the early symptoms of SWD, and the relationship to disease warrants further investigation.

Figure 23.1. **Early stage. Two yellow-brown fronds at base of crown. Villareal.**

Figure 23.2. **Early stage. Internal necrotic zone proximal to the browning of leaflets.**

Figure 23.3. **Early stage. Internal, dry necrotic zone in rachis.**

Figure 23.4. **Early stage. Internal necrosis showing the pale zone externally and central pore, which appears to be the origin of the internal lesion. Note another, similar, pale surface lesion beyond the dark (possibly *Pestalotia*) lesion.**

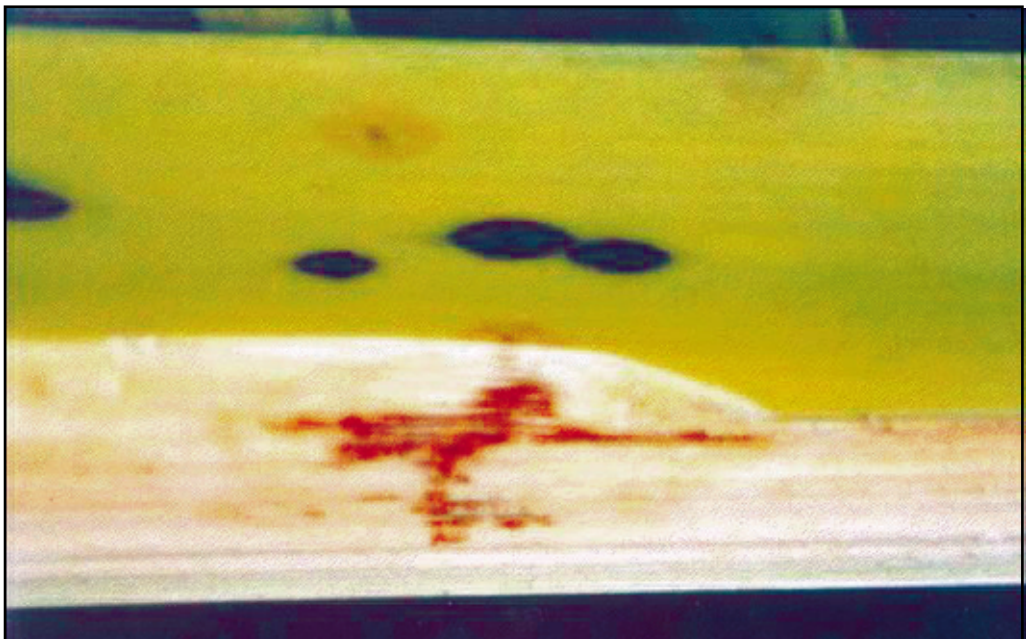

Figure 23.5. **Early stage. Internal lesion showing an affected zone of 'water soaking' in advance of the dark lesion. Note also the longitudinal browning of the vascular bundle traversing the upper portion.**

Figure 23.6. **A putative *Pestalotia* lesion (see also Fig. 23.4) showing a pattern of internal necrosis development that differs from that associated with pale surface lesions. This is not associated with the browning of leaflets on the infected frond.**

Figure 23.7. **Medium stage. Fronds frequently breaking at about mid-point of the rachis, and brown fronds advancing up the crown. Bearing of nuts has ceased.**

Figure 23.8. **Medium stage. Internal part of base of necrotic frond showing necrosis adjacent to that of top of trunk in Fig. 23.9.**

Figure 23.9. **Medium stage. Lesion at junction of base of necrotic frond with top of trunk.**

Figure 23.10. **Base of frond above that in Figure 23.8, showing absence of necrotic zone.**

Figure 23.11. **Medium stage. Internal splitting and browning of vascular bundles starting about 10 cm below meristem and extending downward.**

Figure 23.12. **Medium stage. Browning of fibres below meristematic zone in central trunk; picture shows position in relation to splitting.**

Figure 23.13. Late stage. Basal necrotic zone on one side.

Table 23.3. Association of internal lesions (+ or –) in the rachis with SWD at 2 sites in Socorro.

Site	No. of early-stage SWD tree	Frond no.*	Presence of lesions on browning frond		Presence of lesions on green fronds above
			In proximal green zone	In distal brown zone	
Villareal	1	1	–	+	–
		2	–	+	–
	2	1	–	+	–
		2	–	+	–
	3	1	–	+	
		2	–	+	
	4	1	–	+	
		2	–	+	
Monteverde	1	1	–	+	–
		2	–	+	–
	2	1	–	+	–
		2	–	+	–
	3	1	–	+	
		2	–	+	
	4	1	–	+	
		2	–	+	

* Fronds numbered from oldest living frond.

Recommendations

1. Investigate the nature, structure, pathology and microbiology of the internal lesions in the rachis of early-stage diseased palms. In addition, check rachis tissue from necrotic fronds of palms in areas where SWD does not occur.

   The internal rachis lesions have not been previously reported. Their appearance precedes other changes in diseased palms. Their location is consistent with their causing browning of distal leaflets. They were predominantly found in the distal half of the fronds, and may be associated with the tendency for older fronds of SWD palms to break midway. Standard isolation techniques and light microscopy should be used initially for these studies.

2. Examine the possibilities that a vector is associated with the rachis lesions.

   The common observation of a central hole or pore in the pale lesions that seems to preempt the internal necrosis may indicate that an insect initiates the lesion either by feeding (stylet mouthparts) or by oviposition. The former may leave a stylet sheath, the latter may result in the presence of an egg.

3. Extract soluble components from lesions and test for their phytotoxicity in the types of assays developed in the laboratory of Dr Mendoza at UPLB, and in the DRC laboratory.

   The progression of the disease to mid and late stage could be associated with either the invasion of vascular tissue, causing wilting, or the translocation of toxins, also causing vascular necrosis and wilting. Toxin studies should therefore be done in conjunction with recommendation 1.

4. Subject to the availability of sufficient early-stage palms, a series of tree injection studies should be begun to supplement those done by Dr R. G. Abad.

   The injection method should be modified to that described by Randles et al. (1977), in which three 2–3 cm diameter holes are drilled obliquely downward into the trunk about

1.5 m above ground. Solutions are poured directly into the holes, which are then stoppered.

The solutions to be used:

- Tetracycline (1–2 g active ingredient per tree at one injection time); a response would implicate a mycoplasma.
- Penicillin (1–2 g as above); a response would implicate rickettsia or bacteria.
- Systemic insecticide (e.g. metasystox, dimethoate or aldicarb); a response could implicate an insect or protozoan in the disease.

The tetracycline trial done previously used a very low dose of antibiotic; the trial needs to be repeated at a higher concentration.

Acknowledgments

I am indebted to the staff of Davao Research Centre, especially Dr R. G. Abad and Ms N. C. San Juan, for introducing me to their work on SWD, and for their full cooperation in discussion and field studies of SWD. This work was done during a consultancy with FAO–UNDP. Joana Ferreira facilitated all aspects of this study on behalf of FAO, and I thank numerous other members of PCA, especially Mr C. B. Carpio, Mr R. Blancaver and Dr S. Magat, for their generous assistance in all aspects of this consultancy. I am also most grateful to Dr D. Hanold for providing details of unpublished work on the viroid analyses of SWD-affected tissues from her study in 1990.

24 COCONUT WILT OF CENTRAL KALIMANTAN, INDONESIA

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Introduction

Coconut wilt of Central Kalimantan has been reported by farmers during the past 10 years and seems to be spreading to new locations from its original site near Samuda. Symptoms have been described by B. Zelazny, and are said to closely resemble those of 'Natuna Wilt'. (This similarity could not be investigated owing to difficulties of transport to Natuna Island).

An area near Sampit, Central Kalimantan, Indonesia, was visited on 15 May 1989 to collect coconut leaf samples to be tested in Adelaide for the presence of CCCVd-related and coconut foliar decay virus (CFDV) -related nucleotide sequences. One oil palm with symptoms resembling GOS (see Chapter 18A) was also sampled in the town of Sampit. The objective of this visit was to determine whether either of the pathogens was present and associated with the wilt symptoms.

The collection site was immediately adjacent to a river on swampy ground. Village huts were built among the palms, but there were large amounts of grasses and weeds, including some tall Zingiberaceae. Specimens from these families have been previously shown to contain CCCVd-like sequences in other areas (Hanold and Randles 1991b).

Description of Samples

Resident farmers reported that first symptoms were observed at the river bank, and that subsequently the disease spread inland. It is said to kill the tree in around 4 months. Symptoms have been described previously in detail (Zelazny and Warokka 1990). They did not include chlorosis or clear leaf spots, one or both of which are often part of CCCVd or CFDV symptoms.

The sampled palms were of a 'local' variety and were mostly 10–15 m tall. Seven coconut palms were sampled (Table 24.1) and photographed. The 3 trees in the early stage and the 2 without symptoms were numbered with paint for future reference. The 2 trees in the late stage were cut down. All palms grew on the strip of land between the village road and the river, except for no. 5. Sample no. 9 was a roadside oil palm in a row of about 30 trees, 10–20 years of age, lining a row of houses in the town of Sampit. It was the only one of these palms showing the typical gradient of orange leaf spotting associated with the GOS syndrome (see Chapter 18A) and the presence of CCCVd-related sequences in this species (Hanold and Randles 1989; Randles et al. 1980).

Table 24.1. List of samples

Palm	Symptoms	Sample	Frond	Remarks
Coconut 1	early wilt	1	5	Tree approx. 50 m from palm 3
Coconut 2	early wilt	2	5	Tree approx. 300 m along the road
Coconut 3	early wilt	3	5	Tree approx. 50 m from palm 1
Coconut 4	none	4	5	Tree near palms 1 and 3
Coconut 5	none	5	5	Tree on opposite side of the road
Coconut 6	late wilt	6	2	Erect frond, tree without nuts, cut
		7	4	Hanging necrotic frond of coconut 6
Coconut 7	late wilt	8	2	Tree with few, shrivelled-up nuts, cut
Oil palm 1	orange spots	9	ca. 15	Roadside tree grown for ornamental purpose, Sampit

Method of Analysis

Leaf samples of about 50 g were sealed in plastic, taken to Jakarta, and sent by airfreight to Adelaide. They arrived in good condition and were stored at –20°C.

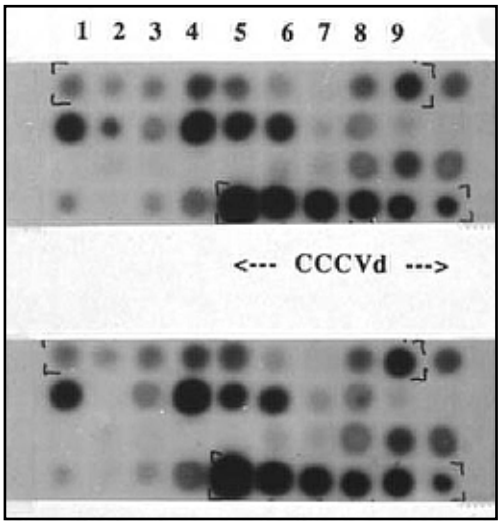
Aliquots were extracted and analysed using the methods described in Chapter 16. The presence of CCCVd-related sequences was tested by both dot blot hybridisation (Fig. 24.1) and PAGE–electroblot hybridisation (Fig. 24.2) with low- and high-stringency washes using the full-length cRNA probe for CCCVd (see Chapter 16).

Extracts were also tested for nucleic acids with sequence homology to coconut foliar decay virus (CFDV) by dot blot hybridisation assay using a ^{32}P-labelled complementary DNA probe of essentially full genome length (Hanold et al. 1988). The post-hybridisation wash was at low stringency.

Results and Discussion

Figure 24.1 shows a dot blot assay of the samples, in which all except one gave a positive signal. The only negative sample, no. 7, was from a necrotic old frond (Table 24.1); all other samples were from living fronds. Samples 2 (early-stage tree) and 6 (late-stage tree) give a slightly weaker signal than the others. Sample 9, from the symptomatic oil palm, was the strongest.

Figure 24.1. **Dot blot hybridisation assay (in duplicate) of samples from Central Kalimantan using full-length cRNA probe for CCCVd (see Chapter 16). Row 1, nos 1, 2, 3: Early wilt symptoms. Row 1, nos 4, 5: Asymptomatic palms. Row 1, nos 6, 7, 8: Late wilt symptoms. Row 1, no. 9: Oil palm with GOS (see Chapter 18A) Rows 2–3: Not connected with this trial Row 4, nos 5–10: CCCVd marker Rows 5–8: Duplicate of rows 1–4.**

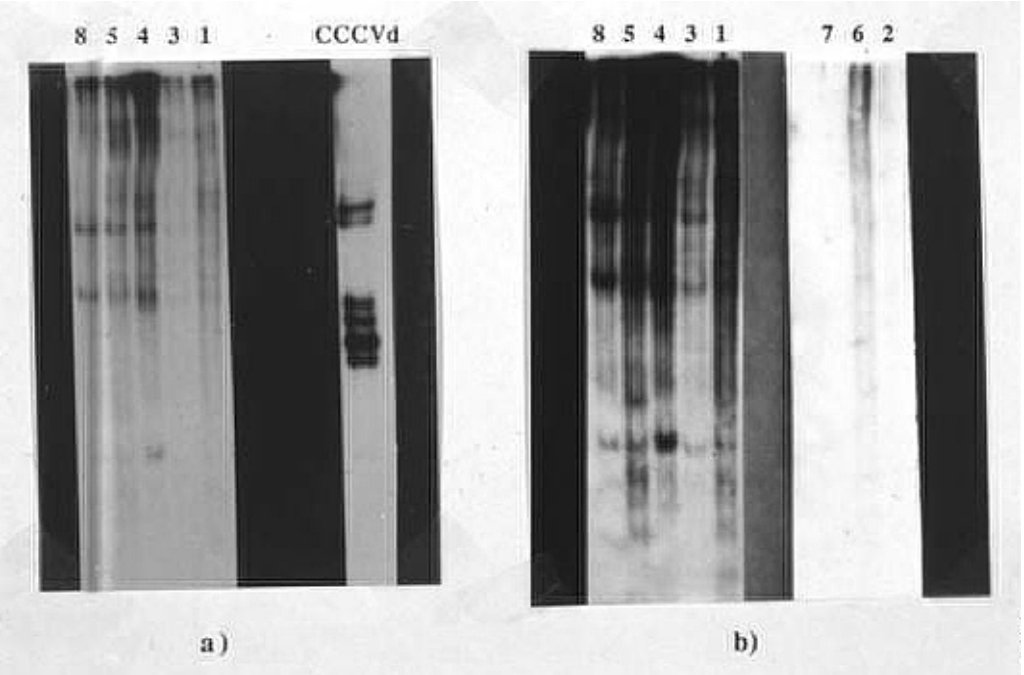


On gel electroblots (Fig. 24.2) it can be seen that samples 1, 3, 4, 5 and 8 contained nucleic acids with sequence homology to the probe, in sizes similar to forms of CCCVd, but that samples 2, 6 and 7 showed no bands. The oil palm (no. 9) showed CCCVd-related bands (data not shown) similar to the ones found in symptomatic oil palms in Solomon Islands (Hanold and Randles 1989, 1991b).

When the stringency of the post-hybridisation wash was increased, probe was lost from the samples more quickly than from the CCCVd marker, suggesting that there was low sequence homology with CCCVd. It should be noted that this is in agreement with our finding that a range of CCCVd-related sequences is present in samples from other Pacific areas without any obvious disease (Hanold and Randles 1991b), as has been described in Chapters 17 and 18.

The dot blot hybridisation assay for CFDV DNA (Hanold et al. 1988) failed to detect any CFDV-related sequences in any of the samples.

Figure 24.2. **Electroblot hybridisation assay from 20% polyacrylamide gels, same probe as Figure 24.1 (low-stringency, post-hybridisation wash for 1 h at 55°C in 150 mM NaCl, 15 mM Na citrate, 0.1% SDS; see Chapter 16 for details). Sample numbers as marked. CCCVd: purified marker viroid. a. Autoradiography for 2 days. b. Autoradiography for 4 weeks.**



Conclusions

1. CCCVd-related nucleic acid molecules were detected in both symptomatic and asymptomatic coconut palms from the wilt area.
2. More extensive work will need to be done to determine whether these nucleic acids are associated with the wilt disease, or whether they are similar to the CCCVd-related sequences already shown to be widespread (Chapters 17 and 18). Isolation and inoculation studies would need to be done to determine whether they are causal, involved in disease induction together with other factors, or not associated with the wilt.
3. CCCVd-related nucleic acids similar to the ones found in Solomon Islands appear to be associated with the typical orange spotting on the oil palm from Sampit.
4. There is no evidence for the presence of nucleic acids homologous to CFDV DNA.

25 BETEL NUT DISEASE OF REEF AND SANTA CRUZ ISLANDS, SOLOMON ISLANDS

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Background and Symptoms

The betel nut disease of Reef and Santa Cruz Islands appeared about 1975, destroying large numbers of betel nut palms. It was thought to be a threat to the local coconut replanting program. A connection with the coconut foliar decay disease of Vanuatu was thought possible, since the group of Reef and Santa Cruz Islands is an extension of the Vanuatu archipelago, and the cixiid *Myndus macfarlani*, a close relative of the vector of CFDV, *Myndus taffini,* has been found there. Symptoms (Figs 25.1–6) have been described by Jackson and Macfarlane (1983) as follows:

> 'Affected palms show mostly single, red, vertical lines of rot, 1–2 cm wide of varying length, below the oldest petiole sheath. These decayed areas, which often have yellow margins, extend internally through the vascular tissue to the pith. Removal of successively younger leaves shows the line of rot gradually narrowing and terminating as a faint red line in the base of a young leaf near the meristem. The latter and tissues beneath appear healthy. Extension of the rot to soil level invariably attracts colonisation by termites. In mature palms inflorescences rot while still in the axils of unopened leaves and those of an older age which have become naturally exposed are shrunken and devoid of nuts.'
>
> On many palms small yellow spots were present, noticeable first on leaves six or seven. On older leaves they become oval in shape with a long axis of 1–2 cm at right angles to the length of the leaflet. Many of the spots showed faint zonation. As the leaves age, the spots coalesce and the entire leaf becomes chlorotic and senesces.
>
> 'Newly developed leaves gradually become shorter until leaf production ceases entirely. Occasionally palms were seen with partially ex[s]erted leaves. Unexposed leaves show various degrees of decay and with cavities filled with copious amounts of gum. Continued deterioration of the internal tissues results in the rot of the bud and the palm dies.'

Photographs provided by courtesy of Dr G. V. H. Jackson.

Results and Conclusions

Thirteen samples were collected by the South Pacific Commission: 9 coconuts, 1 sago palm and 3 betel nuts (Table 25.1). They were extracted and analysed as described in Chapter 16.

The results presented in Table 25.1 show that CCCVd-related bands are present in all the palm samples; this agrees with our findings, reported in Chapters 17 and 18, that some bands are similar to, some different from, other sites, consistent with the situation elsewhere. The number of samples from diseased trees available for analysis is insufficient to show a possible correlation of some of the bands with any specific symptoms or with the betel nut disease.

A dot blot assay (see Chapter 24) using a CFDV probe did not reveal any nucleic acids related to CFDV.

Fig. 25.1. **Frond showing yellow spotting.**

Fig. 25.2. **Close-up of chlorotic leaf spots.**

Fig. 25.3. **Necrosis of flower bud.**

Fig. 25.4. **Non-extension of spear leaf and trunk necrosis.**

Fig. 25.5. **Trunk showing necrotic streaks, made visible by removal of fronds.**

Fig. 25.6. **Close-up of trunk tip with necrotic streaks.**

Table 25.1 **Results of analysis of the betel nut disease of Reef Islands.**

Host	Symptoms	No. sampled	Average number of bands binding CCCVd probe per sample*			
			Overall		Viroid region	
			LS	HS	LS	HS
Coconut	none	8	8.8	3.9	2.6	0.6
Coconut	stunted	1	11.0	6.0	4.0	2.0
Sago palm	leaf spots	1	12.0	4.0	2.0	0
Betel nut	none	1	21.0	9.0	10.0	6.0
Betel nut	dying	2	11.5	4.5	6.0	2.0

* LS = low-stringency, post-hybridisation wash; HS = high-stringency, post-hybridisation wash (see Chapter 16 for details).

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