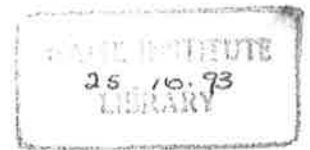

MOLECULAR VARIATION IN COCONUT CADANG-CADANG VIROID (CCCVd)

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SUMMARY

Molecular hybridization with ^{32}P-labelled cRNA and cDNA probes in dot-blots and electroblots and the use of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) demonstrated the presence of multiple coconut cadang-cadang viroid (CCCVd)-like nucleic acids in 11 understorey plant species in coconut plantations. These nucleic acids seemed to be at a lower concentration in their hosts than CCCVd is in coconut. Of the multiple bands found in each plant species, those which were approximately the same size as CCCVd usually had high nucleotide sequence homology with the probe. In contrast, the bands outside the CCCVd region had low homology with the probe. The use of the polymerase chain reaction (PCR) with some of the isolates purified by 2-D PAGE failed to amplify CCCVd-specific nucleic acids under the conditions developed for successful amplification of CCCVd.

The 11 plant species with CCCVd-like sequences are the monocotyledonous hosts *Maranta arundinacea, Alpinia* sp., *Zingiber officinale, Canna flaccida, Commelina diffusa, Commelina benghalensis, Paspalum conjugatum, Brachiaria distachya, Imperata cylindrica, Bambusa blumeana* and the dicotyledonous host *Urena lobata*. The samples were collected at 11 separate sites in five provinces of southern Luzon, Philippines. None showed symptoms which could be associated with the presence of the viroid-like nucleic acids. The incidence in these species was independent of the level of cadang-cadang disease in adjacent coconut plantations, and it is suggested that these plants could act as a reservoir for CCCVd-related nucleic acids in the Philippines.

An unusually severe type of symptom was observed in approximately 12% of 1,787 palms infected by artificial inoculation with preparations of CCCVd. The symptom was first observed in palms 3-7 years after inoculation and is characterized by a loss of leaf lamina, and is associated with severe stunting of the palm and occasional premature death. This symptom has been termed "brooming". PAGE and molecular analyses showed that all palms with brooming had patterns of CCCVd-related nucleic acids which differed from the patterns observed with the common form of cadang-cadang disease. Thus, four bands representing viroids with 246, 247, 296 and 297 nucleotides are observed in extracts from palms with

common cadang-cadang disease. Palms with "brooming" show additional or replacement bands which have electrophoretic mobilities distinct from the above forms of viroid. Bi-directional PAGE was used to separate the brooming-associated nucleic acids from other host nucleic acids. These nucleic acids were eluted as a mixture of the various electrophoretic forms. Three distinct primers were used for reverse transcription of the nucleic acids. The products were amplified by PCR with three pairs of primers. Products with the expected sizes were inserted into the Bluescript plasmid vector by the use of single base A:T overlaps. Twenty-eight full-length clones and 19 half-length clones were obtained, 29 of which were selected and sequenced by fluorescent dye primer cycle sequencing. A number of mutations were observed in the clones, and they fell into the following classes: (i) substitution of C at position 197 of the central conserved domain with either AU, UG, or UU; (ii) substitution of U with A at position 216 in the P domain; (iii) addition of A at position 86 or 87 so that AA became AAA at the boundary of the central conserved region and the V domain; (iv) mutants of varying length resulting from partial sequence duplications of the V and T2 domains commencing from varying positions in the right-hand end of the viroid molecule; and (v) mutations at the boundaries of duplications.

Models were proposed showing optimal secondary structures. These showed that the substitution of C at position 197 of the central conserved domain with either AU or UG could lead to the replacement of a U-loop with a single base pair and that mutations at the boundaries of partial sequence duplications could cause structural changes in the V and T2 domains. The other mutations appeared not to affect secondary structure.

As a mutation at position 197 in the central conserved domain has been previously reported for CCCVd, it is concluded that this site may be important for the control of pathogenicity of CCCVd.

A one-tube, reverse transcription-PCR reaction system was developed for use with CCCVd.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made in the text.

MJB Rodríguez

NAME: Maria Judith B RODRIGUEZ COURSE: Ph.D

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CHAPTER 1

GENERAL INTRODUCTION

1.1 VIROIDS

Viroids are defined as unencapsidated, single-stranded, covalently closed circular, low molecular weight RNAs with extensive regions of intramolecular complementarity (Diener, 1979). The first viroid described was the causal agent for potato spindle tuber disease (Diener, 1971; Gross *et al.*, 1978). Since then, additional plant diseases, most of which were formerly believed to be caused by viruses, have been shown to be viroid-incited (Symons, 1981; Gross *et al.*, 1982; Haseloff *et al.*, 1982; Ohno *et al.*, 1983; Sano *et al.*, 1984; Candresse *et al.*, 1987; Keese *et al.*, 1988; Rezaian, 1990).

1.1.1 Some biological properties

1.1.1a Symptomatology

In some hosts, viroids produce a range of symptoms similar to those observed in viral diseases. Otherwise, they are frequently latent. It has been suggested that viroids and viruses affect the same or similar metabolic pathways in infected cells (Diener, 1987).

The macroscopic symptoms include stunting, epinasty, veinal discoloration, leaf distortions, vein clearing, localized chlorotic or necrotic spots, mottling of leaves, necrosis of leaves and death of the whole plant. The cytopathic effects are chloroplast abnormalities, distortion of cell walls and accumulation of electron-dense deposits. Although viroid infection does not cause disturbances in the synthesis or degradation of host nucleic acids, it induces disruption in the metabolism of growth substances and quantitative changes in host proteins. However, no viroid-coded proteins have been identified in infected cells (Diener, 1987).

1.1.1b Ecology and epidemiology

Most viroids replicate best at relatively high temperatures (30-33°C). High temperature and light intensity are also necessary for rapid symptom expression (Diener, 1987).

All known viroids are transmissible by mechanical means, either readily or with some difficulty. For example, mechanical transmission by contact with farm implements is mainly responsible for the spread of potato spindle tuber viroid (PSTVd) in nature. With other viroids, contaminated budding knives and other tools have been implicated (Diener, 1987).

There have been numerous attempts to identify anthropod vectors of viroids but few positive results have been obtained. An example is the transmission of tomato planta macho viroid (TPMVd) by an aphid species, *Myzus persicae* (Galindo *et al.,* 1989).

Some evidence for transmission of viroid through seed and pollen has been reported. With PSTVd, both seed and pollen transmission have been demonstrated (Diener, 1987; Singh *et al.,* 1992) while evidence of vertical transmission has been shown only with avocado sunblotch viroid (ASBVd) (Diener, 1987).

1.1.2 Classification according to sequence and structure

Sixteen distinct viroids which have been sequenced are listed in Table 1.1. Their secondary structures have been predicted to give linear, rod-like structures. A classification system has been established in which viroids are divided into two main groups based on their conserved core sequences. ASBVd is so far the only member of one group, whereas the rest of the viroids are further divided into two subgroups - B_1, PSTVd subgroup and B_2, ASSVd subgroup. ASBVd does not contain either the PSTVd or the ASSVd core sequence and has a higher A+U (62%) content than the other viroids. Furthermore, it has a self-cleavage site. *In vitro*, in the presence of divalent metal ions such as Mg^{+2} or Ca^{+2}, multimers of ASBVd have the ability to self-cleave into linear monomers. Members of the PSTVd and ASSVd subgroups are easily distinguished by their conserved core sequence, with the PSTVd subgroup having a U-bulged helix (Koltunow and Rezaian, 1989).

Table 1.1 Classification of viroids[a] according to groups and subgroups

Designation	Abbreviation	Length (nucleotides)
A, ASBVd group		
ASBVd subgroup		
Avocado sunblotch viroid	ASBV	246-251
B, PSTVd group		
B1, PSTVd subgroup		
Chrysanthemum stunt viroid	CSVd	354 and 356
Citrus exocortis viroid	CEVd	370-375
Coconut cadang-cadang viroid	CCCVd	246 and 247
Coconut tinangaja viroid	CTiVd	254
Columnea latent viroid	CLVd	370
Cucumber pale fruit viroid[b]	CPFVd	303
Hop latent viroid	HLVd	256
Hop stunt viroid	HSVd	297-303
Potato spindle tuber viroid	PSTVd	359
Tomato apical stunt viroid	TASVd	360
Tomato planta macho viroid	TPMVd	360
B2, ASSVd subgroup		
Apple scar skin viroid	ASSVd	330
Australian grapevine viroid	AGVd	369
Grapevine yellow speckle viroid	GYSVd	367
Grapevine viroid 1B	GVd1B	363

[a] Classification scheme of Koltunow and Rezaian (1989) for viroids that have been sequenced.
[b] Cucumber pale fruit viroid is really a sequence variant of hop stunt viroid.
Ref: Symons, 1991.

1.1.3 Structural domains

By comparative pairwise sequence analysis of members of the PSTVd subgroup, a model of viroid structure has been developed in which five structural domains are distinguished (Fig. 1.1, Keese and Symons, 1985, 1987; Keese *et al.*, 1988). This model also applies to the ASSVd subgroup (Koltunow and Rezaian, 1989). The conserved central domain which is centered around the strictly conserved bulged helix, is considered to be an important control region in viroid replication by signaling functional change through structural alterations. The pathogenic domain is characterized by an oligo ($A_{5\text{-}6}$) sequence present in all PSTVd-like viroids and is associated with symptom expression. The variable domain shows the greatest sequence variability between closely related viroids while in the terminal domains, intermolecular RNA exchange between viroids can take place to give rise to new, chimeric viroid species (Keese *et al.*, 1988).

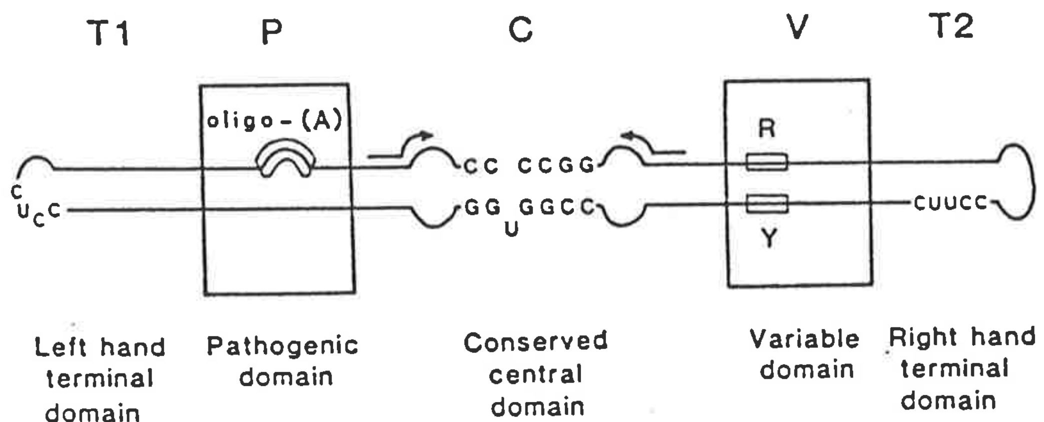


Fig. 1 Model of viroid domains for the potato spindle tuber viroid (PSTVd) group of viroids. The five domains, T1, P, C, V, and T2, were determined from sequence homologies between the viroids. The arrows depict an inverted repeat sequence that can form a stem loop. R and Y indicate a short oligopurine-oligopyrimidine helix. This figure is modified from Keese and Symons (1987) according to Symons (1991).

1.1.4 *In vivo* mutations and recombinations

The natural occurrence of several viroids as two or more variants has been very helpful in defining the roles of the conserved and variable features of the viroid molecule. For the 17 sequence variants of CEVd, mutations are most frequently located in the P and V domains. These variants form two classes of sequence which differ by a minimum of 26 nucleotides in a total of 370 to 375 residues. These two classes correlate with two biologically distinct groups when propagated on tomato plants where one produces severe symptoms and the other gives rise to mild symptoms (Visvader and Symons, 1985).

Eight field isolates of PSTVd of different virulence differ from each other only in a few nucleotides in the P and V domains. The sequence variants are placed into four groups: mild, intermediate, severe and lethal. The nucleotide changes are compared to the mild reference sequence. The intermediate and severe isolates show changes at only three and one sites, respectively, whereas two lethal isolates show changes at either six or seven sites. Despite insertions and deletions, the chain length of 359 nucleotides is strictly conserved in the eight isolates (Schnölzer *et al.*, 1985). The sequence analysis of five new field isolates of PSTVd revealed, however, that the length of their RNA chain is not strictly conserved to 359 nucleotides and that it may vary, so far, between 356 and 360 nucleotides. In comparison with the previously sequenced and least virulent strain as a standard, the two new mild strains differ from it by one or two nucleotides. The new intermediate and severe strains differ from the standard by eight and nine nucleotides, respectively, whereas the new severe to lethal strain differs in seven nucleotides. Most of these mutations are located within the P and V domains. Only two strains have been found in which a single mutation occurs in the right terminal domain (Herold *et al.*, 1992). A strain which was isolated from pepino (*Solanum muricatum*) plants has been found to exhibit major sequence differences from those isolated from potato. When compared to the standard mild strain, this new strain which is 356 nucleotides long has three insertions, six deletions and 14 nucleotide exchanges scattered mostly in the P and V domains, with three of the exchanges in the T_2 domain (Puchta *et al.*, 1990). Also, in the two mild strains of PSTVd isolated from wild *Solanum* sp., one contains two nucleotide

substitutions within the C domain while the other one is identical to the mild strain of PSTVd used as a standard (Owens *et al.*, 1992).

Although CPFVd is six residues longer than HSVd, it is considered to be a sequence variant of HSVd because the two viroids have greater than 90% homology. The nucleotide differences between HSVd and CPFVd are scattered throughout the molecule with the greatest concentration in the P domain. Only two sequence variants of CSVd have been sequenced and most of the sequence changes also occur in the P and V domains (Keese *et al.*, 1988).

The domain model also led to the proposal that the evolution of viroids involved the rearrangements of domains between viroids infecting the same cell followed by further evolution (Keese and Symons, 1985). Experimental support of such a model is difficult to obtain, but new viroid sequences that continue to appear provide indirect evidence (Symons, 1991). For example, in the CLVd sequence, the T_1 and T_2 domains show high sequence homology to the same domains in PSTVd and TASVd, respectively (Hammond *et al.*, 1989). The presence of subdomain lengths of sequences of TASVd in the P domains of PSTVd and of HSVd in the C domain indicate that rearrangements can occur within a domain as well as at the boundaries (Symons, 1991). In AGVd, nearly all the 370 nucleotides appear to be derived from segments of CEVd, PSTVd, ASSVd and GYSVd (Rezaian, 1990). The citrus bent leaf viroid (CBLVd) consisting of 318 nucleotides is constituted from a part of the C domain of the ASSVd subgroup and part of the P and T_1 domains of CEVd. It is the first member of the ASSVd subgroup infecting citrus plants to be characterized (Ashulin *et al.*, 1991). The first viroid to assume a branched conformation when it is folded in the model of lowest free energy is the pear blister canker viroid (PBCVd) of 315 nucleotides. Although it has a conserved sequence located in the T_1 domain of the PSTVd subgroup, it is more closely related to the ASSVd subgroup (Hernandez *et al.*, 1992). The mechanism of these RNA rearrangements is unknown. One probable mechanism is discontinuous transcription where an RNA polymerase copying one viroid changes over to copy a juxtapositioned second template at some point, most likely determined by the tertiary structures of the two templates (Keese and Symons, 1985, 1987).

1.1.5 *In vitro* mutational analysis of structure/function relationships

The success in preparing infectious cDNA clones or their respective RNA transcripts (Cress *et al.*, 1983; Tabler and Sanger, 1984; Meshi *et al.*, 1984; Meshi *et al.*, 1985; Tabler and Sanger, 1985; Ishikawa *et al.*, 1985; Candresse *et al.*, 1990) permits the investigation of structure/function relationships in viroids by site-directed mutagenesis of the cDNAs, as well as construction of viroid chimeras, followed by bioassay. Recently, *in vitro* synthesis of an infectious unit length viroid without resorting to cloning procedures has been demonstrated. This will also allow routine application of oligonucleotide-directed mutagenesis to the study of viroids (Ridgen and Rezaian, 1992).

Mutagenesis has provided new insights into structural features responsible for pathogenicity and host range (Owens and Hammond, 1990). Symptom production by novel intraspecific and interspecific chimeras is controlled by the source of the pathogenicity domain (Visvader and Symons, 1986; Owens *et al.*, 1990). In these experiments, intraspecific recombinants were constructed by exchanging the left and right sides of two sequence variants of CEVd (mild and severe strains) while interspecific chimeras were constructed by similar manipulations involving TASVd and CEVd. Recently, speculation about the mechanism of viroid pathogenesis has centred upon the thermodynamic stability of a virulence-modulating region within the PSTVd pathogenicity domain (Schnölzer *et al.*, 1985) and the ability of nearby nucleotides to base-pair with the 5'-terminus of a 7S host RNA that may be involved in protein translocation (Haas *et al.*, 1988). However, factors other than thermal stability of the virulence-modulating region appear to be important for symptom expression (Visvader and Symons, 1985; Owens and Hammond, 1990). Nonlethal alterations in the pathogenicity domain appear to be clustered in areas which exhibit natural sequence variation (Owens and Hammond, 1990). Sano *et al.* (1992) have demonstrated that T_1, P, V and T_2 domains of TASVd and CEVd contain three discrete regions of sequence and/or structural variability in which variation is correlated with changes in viroid pathogenicity.

With respect to replication mechanisms, *in vitro* mutagenesis has highlighted the importance of the upper portion of the central conserved region for cDNA infectivity (Visvader *et al.*, 1985; Owens *et al.*, 1986; Hammond and Owens, 1987; Candresse *et al.*, 1990; Owens

et al., 1991). This result has been confirmed by studies of the conversion of multimeric PSTVd RNA transcripts into circular monomers by nuclear extracts (Tsagris *et al.*, 1987). Mutagenesis has also been used to detect the presence of alternative sites for the *in vivo* processing of multimeric viroid RNAs (Hammond *et al.*, 1989).

1.1.6 Mechanism of replication

The site of viroid synthesis in the cell is unknown. In PSTVd and CEVd, the nucleolus is a major site of viroid accumulation but nothing is known about the actual site of synthesis (Riesner, 1987). Since viroids do not function as mRNAs, they must be replicated by pre-existing host enzymes (Diener, 1991). The presence of oligomeric viroid forms (usually of opposite polarity) in infected plants suggests that replication occurs by a rolling circle-type mechanism (Branch and Robertson, 1984; Symons *et al.*, 1985). In one of the two variations of the rolling circle mechanism, the infections circular (+ sense) RNA is copied continuously by an unidentified RNA-dependent RNA polymerase* to form a concatameric (-) strand. Specific cleavage of this strand produces monomers that are circularized by a host RNA ligase and then copied by the same or a different RNA polymerase. Specific cleavage of the long linear (+) strand produces monomers that are circularized to give the progeny RNA. ASBVd most likely follows this pathway (Symons, 1991). In the other variation, the linear (-) strand is not cleaved but copied directly to give a linear (+) strand that is cleaved to produce monomers and finally, the circular progeny. Replication of most viroids probably follows this route (Symons, 1991).

1.1.7 Control measures

Most control measures used in agricultural practice are based on prevention rather than cure. These include growing of crops from viroid-free seeds or planting stock and measures to stop these pathogens from entering and spreading through crops (for example, decontamination of tools with a hypochlorite solution). Separation of viroid-infected seeds or propagation material from healthy ones requires diagnostic tests of adequate sensitivity, specificity and rapidity (Diener, 1987).

***or the DNA dependent RNA polymerase II (Schindler & Muhlbach, Plant Sci. 84,221-229,1992)**

So far, the approach of incorporating genetic resistance factors into the genomes of commercially desirable cultivars has not been successful with viroid diseases. This is apparently because of the absence of identifiable resistance factors in viroid host plants (Diener, 1987).

Interference in symptom expression (i.e. cross-protection) has been demonstrated between strains of PSTVd (Niblett *et al.*, 1978; Branch *et al.*, 1988; Khoury *et al.*, 1988). However, it has not been shown in the other viroid systems. Furthermore, no mild strains have been observed for some viroids.

1.2 COCONUT CADANG-CADANG VIROID (CCCVd)

Several reviews provide a detailed coverage of the nature of the coconut cadang-cadang disease and its viroid agent (Zelazny *et al.*, 1982; Randles, 1987; Randles *et al.*, 1988; Hanold and Randles, 1991; Randles *et al.*, 1992). The following is a brief summary.

1.2.1 Cadang-cadang disease

1.2.1a History, distribution and economic importance

The term "cadang-cadang" which means dead or dying comes from the Bicol dialect of the Philippines and is now used to refer to a premature decline and death of coconut palms in the Philippines. The synonym "yellow mottle decline" is seldom used (Rillo and Rillo, 1981).

The first serious outbreak of cadang-cadang was reported in 1931 on San Miguel Island in Albay Province (Ocfemia, 1937). This was followed by observation of the disease at sites at increasing distances from the Bicol Peninsula, with intervening water or disease-free growing areas (Price, 1971). By the early 80's, it had spread widely in the central Philippines and approximately 30 million coconut palms had been killed (Zelazny *et al.*, 1982). The disease continues to spread and at present, the most southernly site of occurrence is Homonhon Island which poses a threat to the coconut plantations in Mindanao (Pacumbaba and Carpio, 1987).

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 to 8 years to reach full bearing, 10 to 13 years of production may be lost from each diseased site (Randles *et al.,* 1992).

1.2.1b Symptoms and host range

A palm infected with cadang-cadang shows a series of symptoms starting with the bearing of smaller, more spherical and scarified nuts. Then, yellow spots develop in the leaves which become numerous as the disease progresses giving the lower two-thirds of the crown a yellowish appearance. Inflorescences become necrotic, infertile and nut production eventually ceases. Fibres remain attached to the bases of the fronds rather than breaking off as in a healthy palm. Frond production and size gradually decline, leaflets become brittle and death of the palm follows (Zelazny *et al.,* 1982). The time between the appearance of the first symptoms and death of the palm ranges from three to more than 15 years and is, on the average, about 10 years. The early stage lasts an average of two years in 19-30 year-old palms but lasts up to 3.75 years in older palms. The medium stage lasts for an average of just over two years. The late stage to death averages about five years. Rarely, there are palms that become infected before they commence bearing and never bear nuts even though they survive well beyond the age of bearing (Zelazny and Niven, 1980).

Although root deterioration has been reported, studies of histological changes have been concentrated on the leaf. Leaflets from infected palms are thinner and the palisade and mesophyll tissues are disorganized. This hypolasia suggests that the disease induces changes at the cell-differentiation phase of leaf development. In the chlorotic leaf spots, chloroplasts appear vesiculate, they accumulate starch and lamellae become disorganized. Some tannin body accumulation has also been observed in vacuoles (Randles *et al.,* 1992).

The experimental and natural hosts of CCCVd identified so far, are all members of the palm family. The mechanically and naturally infected hosts are *Elaies guineensis* (oil palm) and *Corypha elata* (buri palm). The other successfully inoculated palms are *Areca catechu* (betel nut palm), *Adonidia merillii* (Manila palm), *Chrysalidocarpus lutescens* (palmera),

Oreodoxa regia (royal palm), *Ptycosperma macarthuri* (Macarthur palm) and *Phoenix dactylifera* (date palm). Mechanical inoculation is done using a high pressure injector. Conventional methods of inoculation have not been effective (Imperial *et al.*, 1985; Anon., 1986).

1.2.1c Epidemiology

The cadang-cadang disease is rarely observed in young palms. The disease is usually observed only after palms reach 10 years of age and incidence increases approximately linearly for up to 40 years. Incidence then usually remains constant for older palms (Zelazny and Pacumbaba, 1982). The disease has a scattered and apparently random distribution. The rate of spread is slow and gradual ranging from about 0.1% to 1% p.a. in low and high incidence areas, respectively (Randles *et al.*, 1988). The outward advance of the disease is about 500 m p.a. There is no evidence that it originated at one point (Zelazny, 1979).

The natural mode of spread is unknown. The lack of a specific pattern of disease increase does not allow the source of infection in plantation to be inferred and there are still many questions to be answered before epidemiology is sufficiently well understood for control measures to be developed (Randles *et al.*, 1992).

1.2.2 Diagnostic methods

The detection of two small disease-associated RNAs by Randles in 1975 provided the initial clue to the etiology of cadang-cadang. Characterization of pure preparations by electron microscopy (Randles and Hatta, 1979), nucleotide sequencing (Haseloff *et al.*, 1982) and transmission experiments that demonstrated the infectivity of these RNAs (Randles *et al.*, 1977; Mohamed *et al.*, 1985), finally proved that cadang-cadang is caused by a viroid.

Symptomatology is unreliable for disease diagnosis. Therefore, the polyacrylamide gel electrophoresis (PAGE) technique has been used for routine detection of CCCVd. Several modifications starting from the extraction of nucleic acids to gel staining have been done to simplify and miniaturize the procedure and at the same time improve its sensitivity (Imperial *et*

al., 1981; Imperial and Rodriguez, 1983). Two-dimensional and bi-directional electrophoresis, which have been developed for general use in the detection of viroid-like RNAs (Schumacher *et al.*, 1983; Rivera-Bustamante *et al.*, 1986; Tabler *et al.*, 1989), are useful for identifying CCCVd in mixtures when linear RNAs of similar mobility are present.

CCCVd is detected by PAGE on the basis of size. For a more sensitive and specific assay based on its unique sequence, dot-blot and electroblot molecular hybridization with labelled complementary RNA or DNA probe have been developed (Randles and Palukaitis, 1979; Barker *et al.*, 1985; Imperial *et al.*, 1985; Hanold and Randles, 1991).

1.2.3 Physical properties

In the native state, CCCVd is partially sensitive to the single-strand-specific S1 nuclease (Randles *et al.*, 1976; Randles and Palukaitis, 1979) as expected for a partially base-paired structure. Thermodynamic studies indicate that the native molecules do not adopt significant tertiary folding, but they exhibit highly cooperative melting of base-paired regions at a Tm of 49°C in 10 mM NaCl. An intermediate structure, a hairpin comprising a single-stranded loop of 14 bases and a stem of 9 base pairs, forms at temperatures just about the Tm. The stem melts at about 58°C to produce a completely denatured circle. The short sequence that is exposed as a single-stranded loop occurs in the upper central conserved region common to most of the other viroids (Randles *et al.*, 1982; Riesner *et al.*, 1983).

1.2.4 Variation in the nucleotide sequence

CCCVd with 246 nucleotides is the smallest known viroid. In addition to this basic form, at least three types of sequence variants have been recognized by sequencing isolates from individual palms and from fronds of different age within a single palm. These include an insertion of cytosine at position 197 of the C domain to give a 247-nucleotide sequence variant, duplications of the V and T_2 domains of 41, 50, 55 or 100 nucleotides and mutations of bases adjacent to the boundaries of some of the partial duplications. Detected concurrently with the

monomers are their respective dimers which are covalently linked forms of the monomers, incorporating the same sequence variations (Haseloff *et al.*, 1982; Keese *et al.*, 1988).

1.2.5 Correlation of CCCVd structure with disease progress

Variation in CCCVd is related to the stage of disease development. The small 246/247 variants (with their corresponding dimers) appear early in infection but, as symptoms develop, the large 287/296/297/301/346 variants (also with their corresponding dimers) arise and eventually dominate the viroid population as the disease progresses (Imperial *et al.*, 1981; Mohamed *et al.*, 1982). A systematic but more subtle progression has been observed for the variants containing either one cytosine (246 or 296) or two cytosine (247 or 297) residues at nucleotide position 197. If infection starts with variant 246, 247 appears next followed by 296, then 297. If variant 247 appears first, it is replaced only by 297 (Imperial and Rodriguez, 1983). All variants are infectious and in the field, the frequency of occurrence of $CCCVd_{246}$ in the early-stage palms at four sites was greater than that of $CCCVd_{247}$ (Randles *et al.*, 1992).

The functional implications of the development of larger variants of CCCVd during disease progression are not known. Presumably, it has some advantage in replication, perhaps by providing increased competition for binding to some host component important for replication, but which is in limited supply (Keese *et al.*, 1988).

1.2.6 CCCVd as related to CTiVd and the viroid-like sequences in oil and coconut palms and other monocotyledons in the south-west Pacific

The symptoms of the tinangaja disease of coconuts in Guam are similar to those of the cadang-cadang disease except for the effect on nut production. Whereas the coconuts from palms infected with cadang-cadang become more spherical and scarified, palms afflicted with tinangaja produced mummified nuts with no kernel present (Boccardo *et al.*, 1981). The viroid associated with this disease, CTiVd, is 254 nucleotides long and shares 64% overall sequence homology with CCCVd (Keese *et al.*, 1988).

The presence of small nucleic acids with nucleotide sequences similar to CCCVd has been demonstrated in African oil palm, coconut palm and some other monocotyledonous species in several areas of the south-west Pacific region. The oil palms have orange leaf spots resembling those described for oil palm naturally infected with CCCVd in the Philippines, and also characteristic of a condition known as "genetic orange spotting" (GOS). Preliminary evidence has been provided that GOS is an infectious disorder caused by a viroid. The coconut palms do not show symptoms typical of cadang-cadang disease, but sometimes are chlorotic, stunted, or have a reduced yield. These findings suggest that viroids with nucleotide sequences similar to CCCVd occur widely in palms and other monocotyledons outside the Philippines (Hanold and Randles, 1991).

SCOPE OF THIS THESIS

In the central Philippines where the cadang-cadang disease has been in existence since the 1930's, there is a greater possibility that alternate hosts and naturally occurring variants of CCCVd will be found. Thus, in this thesis, a range of different plants other than palm species which grow naturally in coconut plantations was screened. CCCVd-like molecules were detected in some of the species tested and these were isolated, purified and characterized. Then, in a significant number of palms inoculated with CCCVd, a new type of symptom appeared which was more severe than the typical cadang-cadang symptoms. These palms were analyzed for their viroid pattern by polyacrylamide gel electrophoresis and the viroids were also isolated, purified and further characterized by molecular cloning and nucleotide sequencing.

The detection of CCCVd-like sequences in plants other than palm species shows that they could act as sources of inoculum for coconut palms in the field. Definition of the CCCVd reservoir should lead to a better understanding of the epidemiology of the disease. Furthermore, the identification of a herbaceous host would provide a system that will facilitate studies on CCCVd. On the other hand, the detection of more variants of CCCVd would be useful in identifying its conserved and variable features and their functional implications.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CCCVd

Partially purified CCCVd used as standard marker was prepared according to Section 2.2.3 from infected palms at different stages of the disease in order to obtain the same amount of each of the 246, 247, 296 and 297 sequence variants with their respective dimers.

Pure $CCCVd_{246}$ used for the synthesis of ^{32}P-labelled ss cDNA probe was prepared according to Mohamed *et al.*, (1985).

2.1.2 ^{32}P-labelled cRNA probe

The cRNA probe used in the early part of this study was prepared by Dr JL McInnes of the Plant Science Department of the Waite Agricultural Research Institute (WARI). Using the kit from BRESATEC (Adelaide), transcription was done from a pSP_{64} plasmid containing a monomeric insert of the 246 nucleotide form of CCCVd at the BamH1 site.

2.1.3 Synthetic CCCVd-specific DNA oligonucleotide primers

The primers 5'-d(GGCCTCTCCTGCAGTGGTTTTGGGGTGCCC)-3' and 5'-d(CCACTGCAGGAGAGGCCGCTTGAGGGATCCCC)-3' were kindly provided by Dr W Rohde of the Max Planck Institut für Züchtungsforschung, Köln, Germany.

The primers 5'-d(GAGTTGTATCCACCGGGTAGTCTCC)-3', 5'd(GGTTTCCC CGGGGATCCCTC)-3', 5'-d(AGGGATCCCCGGGAAACCT)-3 and 5'd-(GGAGACTAC CCGGTGGATACAACT)-3' were synthesized by Dr N Shirley of the Plant Science Department, WARI, using the Applied Biosystems 380B DNA synthesizer.

2.1.4 Biochemicals and miscellaneous chemicals

The main biochemicals and miscellaneous chemicals used in this study are listed in Appendix A.

2.1.5 Phenol reagents, polyacrylamide/agarose gels and bacterial media

Preparation of phenol reagents (for RNA and DNA extractions), polyacrylamide/ agarose gels and bacterial media are described in Appendix B.

2.2 METHODS

2.2.1 Isolation of low molecular weight nucleic acids from tissues of plants other than coconut

Fresh or frozen tissues were homogenized with 1.5 vol (w/v) of buffer containing 0.5 M sodium acetate, 10 mM $MgCl_2$, 20% ethanol and 3% SDS, and 1.5 vol (w/v) of water-saturated phenol (Laulhere and Rozier, 1976). The homogenate was incubated with shaking at 37°C for 15 min and mixed vigorously for 10 min with 0.5 vol of chloroform. The aqueous phase recovered by centrifugation at 10,000 rpm for 10 min was re-extracted with 0.5 vol of phenol and 0.5 vol of chloroform (v/v) and again centrifuged as above. The nucleic acids were recovered from the supernatant by precipitation with 0.33% CTAB for 30 min on ice and the resulting pellet was washed twice with a 0.1 M sodium acetate solution in 75% ethanol (Imperial *et al.*, 1985). The precipitate was dried briefly, resuspended in the minimum amount of 0.25 M sodium acetate and extracted with 0.5 vol each of phenol and chloroform. After centrifugation at 10,000 rpm for 10 min, the supernatant was recovered and an equal volume of 4 M LiCl was added. The mixture was incubated at 4°C for 15-18 hr and centrifuged at 10,000 rpm for 10 min at 4°C (Imperial *et al.*, 1981). The LiCl-soluble components were recovered and precipitated with 2.5 vol of ethanol. The pellet was either kept in ethanol at -20°C for long

storage or dried and dissolved in 0.01 M sodium acetate solution with 10% sucrose for immediate assay.

2.2.2 Mini-preparation of low molecular weight nucleic acids from coconut leaves

A 1-g leaf sample was placed in a plastic bag with 1 vol (w/v) of buffer containing 100 mM Na-K phosphate buffer, pH 7, 10 mM EDTA and 0.5% 2-mercaptoethanol. The tissue was crushed with a pestle and the juice was squeezed out into a centrifuge tube containing 0.5 vol each of phenol and chloroform. After vortexing for a few minutes, the mixture was centrifuged at 10,000 rpm for 10 min. The nucleic acids in the aqueous phase were precipitated with CTAB and fractionated with LiCl as in Section 2.2.1 but omitting the phenol/ chloroform extraction in between.

2.2.3 Large-scale preparation of low molecular weight nucleic acids from coconut leaves

Bulk extraction of low molecular weight nucleic acids from coconut leaves was done according to a previously described procedure (Mohamed *et al.*, 1985). Batches (250 g) of chopped leaflets were blended in 750 ml of pre-cooled 0.1 M Na_2SO_3. The slurry was strained through cotton muslin and clarified by centrifugation at 10,000 rpm for 10 min at 4°C. Supernatants from 5 kg of leaf material were pooled and 8% PEG 6000 was added. After 2 hr incubation at 4°C, the resulting precipitate was collected by centrifugation as above. Nucleic acids were extracted from the precipitate by dissolving it in 100 ml of 1% SDS, then adding 0.5 vol each of phenol and chloroform and shaking vigorously for 1 hr. The aqueous supernatant phase was collected after centrifugation and re-extracted twice with 0.5 vol each of phenol and chloroform for 30 min. Further purification steps with CTAB and LiCl were done as in Section 2.2.2.

2.2.4 Viroid purification

2.2.4a Two-dimensional (2-D) or bi-directional polyacrylamide gel electrophoresis (PAGE)

Separation of viroids from contaminating host nucleic acids, either by two-dimensional (2-D) or bi-directional PAGE, was done as described by Schumacher *et al.*, (1983) and Tabler *et al.*, (1989). The first dimension gel was identical for both systems. Partially purified nucleic acid extracts were electrophoresed on a non-denaturing 7% slab gel in TBE buffer (90 mM Tris - 90 mM borate - 2 mM EDTA) (Peacock and Dingman, 1968). The gel was stained with ethidium bromide (500 µg/L) and the zones containing the viroid molecules were cut out as required. Vertical lanes with different viroid samples were cut out separately for 2-D PAGE whereas for bi-directional system, the gel was horizontally cut across all lanes containing the viroid position which was determined with reference to the marker viroid. Each gel slice was placed either below or on top of the second dimension 7% gel with 8 M urea. The circular and linear viroid forms were located by ethidium bromide staining (as above) and then excised from the gel.

2.2.4b Recovery of the viroids from polyacrylamide gel

Where gels of less than 1 mm thickness were used, the viroids were efficiently eluted by soaking the intact gel pieces for 14-16 hr at 37°C in 0.5 M ammonium acetate, 1 mM EDTA, pH 8 and 0.1% SDS (Sambrook *et al.*, 1989). The elution buffer was removed, extracted once with 0.5 vol each of phenol and chloroform and centrifuged at 10,000 rpm for 10 min. The aqueous phase was recovered and 2.5 vol of ethanol was added to precipitate the viroids.

Where the gel thickness was more than 1 mm, the viroids were electroeluted (Maniatis *et al.*, 1982) by placing the excised band in a dialysis bag with 0.5 x TBE buffer. The bag was closed and immersed in a tank filled with electrophoresis buffer. Current was passed through the bag for 2-3 hr at 100 V to elute the viroids out of the gel and onto the inner

wall of the bag. The polarity of the current was reversed for 2 min to release the viroids from the bag wall. The gel buffer was removed from the bag and extracted once with phenol and chloroform (as above). The viroids were precipitated with ethanol in the presence of 0.3 M sodium acetate.

2.2.5 PAGE assay

Nucleic acid samples resuspended either in 0.01 M sodium acetate with 10% sucrose or in 0.5% bromophenol blue/xylene cyanol in TBE with 50% glycerol (v/v) were applied to a non-denaturing polyacrylamide gel containing 20% acrylamide-bisacrylamide in the ratio of 99:1 (Imperial and Rodriguez, 1983). Electrophoresis was at 250 or 125 V for 6 or 12 hr, respectively, in TBE buffer. The gel was stained with silver nitrate according to Sammons *et al.* (1981) but with the following modifications: the gel was fixed in 10% trichloroacetic acid for 15 min (Imperial *et al.*, 1985), followed by two brief rinses with glass-distilled water, then soaked (with shaking) in 0.18% silver nitrate for 1 hr. The gel was again washed two to three times with water and soaked (with shaking) in reducing solution (87.5 mg/L of sodium borohydride and 0.75% formaldehyde in 0.25 M NaOH) until the bands were visualized. The bands were enhanced by incubating the gel in 0.75% sodium carbonate with 5% acetic acid added to prevent the gel from expanding.

2.2.6 Molecular hybridization assay

2.2.6a Synthesis of ^{32}P-labelled single-stranded complementary DNA (^{32}P-ss cDNA) probes

Synthesis of ^{32}P-ss cDNA probes was carried out in a reaction volume of 30 µl containing 15 mM Tris-HCl, pH 8.3, 40 mM KCl, 0.85 mM $MgCl_2$, 0.05% mercaptoethanol, 50 µM each of dATP, dGTP and dTTP, 30 µCi of [α-^{32}P) dCTP (≅ 3000 Ci/mmol), 1 µg of CCCVd-specific primer 5'-d(GAGTTGTATCCACCGGGTAGTCTCC)-3' or 5'-d(GGCCT CTCCTGCAGTGGTTTTTGGGGTGCCC)-3', 1 µg of purified $CCCVd_{246}$ and 200 units of

M-MuLV reverse transcriptase. The template viroid and the primer with the buffer solution were first incubated at 90°C for 3 min, then at 55°C for 3 min, after which the reverse transcriptase and ^{32}P-dCTP were added and the whole reaction mixture was incubated at 37°C for 4-5 hr. The reaction was stopped by adding 20 mM EDTA. The mixture was then chromatographed on a G50 (fine) - Sephadex column (1.5 ml vol in a 2 ml Pasteur pipette) with sterile TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA). Approximately 400 µl of TE buffer was added each time to collect at least 6 fractions. Usually, fractions 2 and 3 contained the ^{32}P-cDNA while the rest of the fractions contained the unincorporated ^{32}P-dCTP. Radioactivity of the fractions was determined by Cerenkov-counting and those containing the ^{32}P-cDNA were pooled and extracted once with 1 vol each of phenol and chloroform. After centrifugation at 10,000 rpm for 2 min, the aqueous phase was recovered and an equal volume of formamide was added. The mixture was heated at 100°C for 2-3 min, then chilled on ice before use. The specific activity was ca. 2×10^7 cpm/µg.

2.2.6b Preparation of dot-blot/electroblot

Nucleic acid samples of 1 µl were dotted directly onto a sheet of nylon membrane (Zeta-probe, Biorad) which had been washed with distilled water and air dried. After application of the samples, the membrane was baked for 2 hr at 80°C (Thomas, 1980).

Nucleic acid samples and a CCCVd standard marker were fractionated by electrophoresis in 20% polyacrylamide gel buffered with TBE. After completion of the run, the gel was equilibrated in TAE (9 mM Tris, 4 mM Na-acetate, 0.4 mM EDTA, pH 7.4) for 15 min and assembled in contact with the nylon membrane in an electroblot apparatus filled with TAE buffer. Current was passed through the gel at 60 V, 0.6 A for 5 hr or at 30 V, 0.3 A for 16 hr followed by 60 V, 0.6 A for 1 hr to transfer the nucleic acids to the nylon membrane (Hanold and Randles 1991). The membrane was baked for 2 hr at 80°C.

2.2.6c Hybridization

The nylon filters were prehybridized for 15-20 hr at 42°C in a buffer made according to Thomas (1980) but with some modifications recommended by the manufacturer of the nylon membrane (Biorad): 50% deionized formamide; 5 X SSC (750 mM NaCl, 75 mM Na-citrate); 50 mM sodium phosphate buffer, pH 6.5; 5 mm EDTA; 0.2% SDS; 1 mg/ml denatured carrier DNA and 0.2% each of bovine serum albumin (BSA), Ficoll 400 and polyvinylpyrrolidone (PVP) of mol wt 40000.

The denatured cDNA or cRNA (Section 2.1.2) probe was added to the hybridization mixture at approximately 10^6 cpm/ml. The hybridization mixture consisted of the modified prehybridization buffer (except that 250 µg/ml denatured carrier DNA and 0.02% each of BSA, Ficoll 400 and PVP 40000 were used) and 10% dextran sulphate. Hybridization was done at 42°C for 20-40 hr (Thomas, 1980). For washing at low stringency, the filters were immersed in 0.5 X SSC, 0.1% SDS for 5 min at 20°C, then washed with agitation in 0.1 X SSC, 0.1% SDS for 2 hr at 55°C and autoradiographed at -70°C using an intensifying screen. For washing at high stringency, the filters were re-washed with agitation at 65°C for 2 hr in the same buffer as above and again autoradiographed regulating the exposure times in order to obtain signal intensities similar to those of the low stringency wash (Hanold and Randles, 1991).

2.2.7 Amplification of viroids by polymerase chain reaction (PCR)

2.2.7a Preparation of template

First strand cDNA synthesis using M-MuLV reverse transcriptase. This was done as in Section 2.2.6a except that dCTP was added at the same concentration as the other dNTPs and no ^{32}P was incorporated in the reaction mixture. The cDNA was precipitated from the reaction mixture by adding 2.5 vol of ethanol and incubating the mixture at -20°C for at least 30 min. The precipitate was recovered by centrifugation at 15,000 rpm for 20-30 min, dried briefly and resuspended in deionized water.

First strand cDNA synthesis using AMV reverse transcriptase. This was done according to Ding, SW (unpublished). The viroid RNA and the primer (1 μg each) resuspended in deionized water were incubated at 80°C for 12 min and chilled on ice for 5 min. The components (50 mM Tris-HCl, pH 8.3; 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine; 40 units of RNAsin; 10 mM DTT and 1 mM each of dATP, dCTP, dGTP and dTTP) required for reverse transcription were added to a final volume of 25 μl. The reaction was started by adding 25 units of AMV reverse transcriptase and incubating the mixture at room temperature for 5 min, then at 55°C for 30 min. The mixture was extracted once with 1 vol each of phenol and chloroform and centrifuged at 10,000 rpm for 5 min. The cDNA was recovered from the aqueous phase by ethanol precipitation (2.5 vol) at -20°C for at least 30 min followed by centrifugation at 15,000 rpm for 20-30 min. The dried pellet was dissolved in deionized water and stored at -20°C.

2.2.7b PCR reaction

The buffer solution (as provided in the PCR kit manufactured by Promega) contained the following: 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.01% gelatin and 1.25 mM each of the four dNTPs. Approximately 250 ng each of the two primers and the first strand cDNA were added to the buffer solution. The *Taq* polymerase (1.28 units) was added to a final volume of 25 μl only after the initial denaturation of the above mixture at 96°C for 3 min. The mixture was overlaid with mineral oil. Amplification was for 40 cycles on a Hybaid thermal cycler under the following conditions: 93°C for 45 sec, 55°C for 45 sec and 72°C for 3 min. An extra elongation time at the end of the cycles was done at 72°C for 15 min.

2.2.8 Molecular cloning of PCR products

2.2.8a Purification of PCR products and preparation of the cloning vector

The PCR mixtures, after termination of the reaction, were fractionated on a 7% non-denaturing polyacrylamide gel in TBE buffer. The gel was stained with ethidium bromide (500 μg/L) and the zones containing the ds cDNAs of the desired lengths as determined by comparison with a DNA mol. wt. marker were cut out. The ds cDNAs were recovered from the gel as described in Section 2.2.4b and resuspended in deionized water.

The cloning vector was prepared according to Marchuk *et al.* (1991) and Holton and Graham (1991). About 1 μg of Bluescript SK^+ plasmid (Stratagene) was digested with EcoRV (5-10 units/10 μl vol) in the presence of the appropriate restriction buffer (provided by the manufacturer, Boehringer) at 37°C for 2 hr. The mixture was extracted once with 1 vol each of phenol and chloroform, centrifuged at 10,000 rpm for 5 min and the blunt-ended vector was recovered from the aqueous phase by ethanol precipitation (2.5 vol) at -20°C for at least 30 min. The pellet was dried briefly, dissolved in 10 μl of deionized water and incubated for 2 hr at 70°C with *Taq* polymerase (5.5 units/μg plasmid/20 μl vol) in the presence of standard buffer conditions (Section 2.2.7b) except that only 2 mM dTTP was added. The absence of any other nucleotides in the reaction resulted in the addition of a single thymidine at the 3' end of each fragment. The mixture was extracted once with 1 vol each of phenol and chloroform and centrifuged at 10,000 rpm for 5 min. The T-vector was recovered from the supernatant by ethanol precipitation (2.5 vol) at -20°C for at least 30 min. The dried pellet was dissolved in deionized water.

2.2.8b Ligation of ds cDNA to the T-vector

The ligation reaction (Sambrook *et al.*, 1989; Mead *et al.*, 1991) was done in a 10 μl buffer solution containing 66 mM Tris-HCl, pH 7.5 at 20°C; 5 mM $MgCl_2$; 1 mM DTT; 1 mM ATP; 1 unit T4 DNA ligase (Weiss unit) and 20-25 ng of the T-vector. The ds cDNA was added to maintain an approximate vector to insert ratio of 1:1 and 1:3. This was achieved by

estimating the amount of insert DNA by gel electrophoresis in comparison with a standard of known concentration. The ligation mixture was incubated overnight at 5-16°C.

2.2.8c Preparation of competent cells

E. coli JM109 competent cells were prepared according to Hanahan (1983) and Sambrook *et al.* (1989). A single colony of *E. coli* JM109 was picked from a streak plate and grown overnight in 3 ml of LB medium. Approximately 50 ml SOB medium containing 20 mM $MgSO_4$ in a 1 litre flask was inoculated with 500 µl of the overnight culture. The cells were grown (with shaking) at 37°C until the OD_{600} was about 0.45-0.55 (approximately after 2.5 to 3 hr incubation). For efficient transformation, it is essential that the number of viable cells should not exceed 10^8 cells/ml. Thus, to monitor the growth of cells, OD_{600} was determined every 20-30 min. The culture was placed on ice for 10-15 min. The cells were pelleted by centrifugation at 2,500 rpm for 12 min at 4°C and resuspended in 1/3 vol TFB (10 mM MES, pH 6.3; 45 mM $MnCl_2{\cdot}4H_2O$; 10 mM $CaCl_2{\cdot}2H_2O$; 100 mM KCl and 3 mM hexamine cobalt chloride) by gently vortexing or sucking up and down with a Pasteur pipette. The suspension was placed on ice for 10-15 min and the cells were pelleted again at 2,500 rpm for 12 min at 4°C. The pellet was resuspended in TFB at 10/125 of the original volume of the cells. DMS0 was added to 3.5%, the mixture swirled and left on ice for 5 min. Then DTT was added to 75 mM, the mixture swirled and left on ice for 10 min. DMS0 was again added to 3.5%, the mixture swirled and left on ice for 5 min. Finally, the suspension was dispensed as 200 µl aliquots into chilled 1.5 ml tubes.

2.2.8d Transformation of the competent cells with the recombinant plasmids

Transformation of the competent cells was done according to Hanahan (1983). The ligation mixture (Section 2.2.8b) containing the recombinant plasmids was added to 200 µl of competent cells in a chilled 1.5 ml tube. The mixture was swirled, incubated on ice for 30 min, heated at 42°C for 2 min and immediately placed on ice for 2 min. Then, 800 ml of SOC

medium (7 µl 50% glucose/ml SOB) was added and mixed by inversion. The mixture was transferred to a culture tube and incubated at 37°C for 45 min to allow the cells to recover.

2.2.8e Selection for recombinants

As described by Sambrook *et al.* (1989) and Titus (1991), a 200 µl aliquot of the transformation mixture was spread gently (using a bent Pasteur pipette) on an LB plate with 60 µg/ml of ampicillin, 0.5 mM IPTG and 40 µg/ml X-gal: The rest of the mixture was concentrated by centrifugation at 10,000 rpm for 1 min. The supernatant was poured off and the cells resuspended in 200 µl of LB medium were also plated as above. The plates were incubated at 37°C for 14-16 hr to establish colonies. Recombinant colonies were white.

2.2.9 Mini-preparations of recombinant plasmids

Extractions of plasmid DNA were done by the alkaline lysis method (Sambrook *et al.*, 1989; Applied Biosystems User Bulletin 18, 1991).

White colonies were picked from the plate and each transferred into 3 ml LB medium containing 60 µg/ml of ampicillin. The cultures were incubated overnight at 37°C with vigorous shaking. A 1.5 ml aliquot of each culture was pelleted in a microcentrifuge tube by centrifugation at 10,000 rpm for 1 min at room temperature. The supernatant was removed by aspiration and the bacterial pellet was resuspended (by pipetting up and down) in 200 µl of ice-cold buffer solution containing 50 mM glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA, pH 8.0. About 300 µl of freshly prepared 0.2 NNaOH-1% SDS was added and the contents of the tube were mixed by inversion before incubating on ice for 5 min. The use of a vortex mixer was avoided so as to minimize shearing of the contaminating chromosomal DNAs. The solution was neutralized by adding 300 µl of ice-cold 3 M potassium acetate, pH 4.8. The contents of the tube were mixed by inversion and then incubated on ice for 5 min. Cellular debris was removed by centrifugation at 10,000 rpm for 10 min at room temperature. The supernatant was transferred to a clean tube. RNase A (DNase-free) was added to a final concentration of 20 µg/ml and the tube was incubated at 37°C for 20 min. Extraction with 400

μl of chloroform was done twice by mixing the layers by hand for 30 sec after each extraction. After centrifugation at 10,000 rpm for 5 min, the aqueous phase was removed to a clean tube. Total DNA was precipitated by adding an equal volume of 100% isopropanol and immediately centrifuging the tube at 10,000 rpm for 10 min at room temperature. The DNA pellet was washed with 500 μl of 70% ethanol, dried under vacuum for 3 min, resuspended in 20 μl of deionized water and stored at -20°C.

2.2.10 Analysis of the sizes of inserts in recombinant plasmids by restriction enzyme digestion and PAGE

The recombinant plasmids were analyzed for the presence of the correct size of insert DNA by digestion with EcoRI and HindIII according to the manufacturer's directions (Boehringer). The reaction mixtures were then fractionated on a 20% non-denaturing polyacrylamide gel in TBE buffer using a 123 DNA ladder as the mol wt marker.

2.2.11 Fluorescent dye primer cycle sequencing

2.2.11a Preparation of dsDNA template

The template plasmid DNAs were prepared as in Section 2.2.9 and further purified by PEG precipitation (Applied Biosystems User Bulletin 18, 1991). The plasmid pellet was dissolved in 32 μl of deionized water. Then, 8 μl of 4 M NaCl and 40 μl of sterile 13% PEG 8000 were added. After thorough mixing, the solution was incubated on ice for 20 min and the plasmid DNA was pelleted by centrifugation at 10,000 rpm for 15 min at 4°C in a fixed-angle rotor. The supernatant was removed carefully and the pellet was rinsed with 500 μl of 70% ethanol. The pellet was dried under vacuum for 3 min, resuspended in 20 ml of deionized water and stored at -20°C.

2.2.11b Cycle sequencing reaction

Cycle sequencing of the dsDNA was done using the *Taq* dye primer cycle sequencing kit manufactured by Applied Biosystems Inc (ABI) and the recommended protocol (ABI dye primer cycle sequencing/Part no. 901482/Rev. B).

The following reagents were aliquoted into four 0.5 ml tubes:

Reagent	A	C	G	T
d/dd NTP mix	1 µl	1 µl	2 µl	2 µl
Dye primer (0.4 pmol/µl)	1 µl	1 µl	2 µl	2 µl
5X cycle seq. buffer (400 mM Tris-HCl, pH 8.9 at RT; 100 mM $(NH_4)_2SO_4$; 25 mM $MgCl_2$)	1 µl	1 µl	2 µl	2 µl
DNA template (200-250 ng/µl)	1 µl	1 µl	2 µl	2 µl
Diluted *Taq*	1 µl	1 µl	2 µl	2 µl
Total volume	5 µl	5 µl	10 µl	10 µl

The d/ddNTP mixtures contained the following:

A: 1.5 mM ddATP; 62.5 µM dATP; 250 µM dCTP, 375 µM C^7dGTP and 250 µM dTTP

C: 0.75 mM ddCTP; 250 µM dATP; 62.5 µM dCTP; 375 uM C^7dGTP and 250 µM dTTP

G: 0.125 mM ddGTP; 250 µM dATP; 250 uM dCTP; 94 uM C^7dGTP and 250 µM dTTP

T: 1.25 mM ddTTP; 250 µM dATP; 250 µM dCTP; 375 uM C^7dGTP and 62.5 µM dTTP

The diluted *Taq* was prepared by mixing 0.5 µl of AmpliTaq® DNA polymerase (8 U/µl); 1.0 µl of 5X cycle sequencing buffer and 5.5 µl of deionized water.

The four reaction tubes above were overlaid with 30 µl mineral oil and placed in a thermal cycler (PTC-100^{TM} Programmable Thermal Controller, MJ Research, Inc). The cycling was started as follows: 95°C for 30 sec, 55°C for 30 sec and 70°C for 1 min for 15 cycles. Then it was continued for another 15 cycles at 95°C for 30 sec and 70°C for 1 min. At the completion of the run, the temperature was rapidly dropped to 4°C. For best results, the total time required for 30 cycles should be 1 hr and 45 min ± 5 min.

The extension reaction mixtures from the four tubes were pipetted out and combined into a 1.5 ml tube containing 80 µl 95% ethanol with 1.5 µl 3 M sodium acetate, pH 5.3. The tube was placed on wet ice for 10-15 min and centrifuged at 15,000 rpm for 15-30 min. The supernatant was carefully discarded. The pellet was rinsed with 250 µl of 70% ethanol, dried *in vacuo* for 1-3 min and resuspended in 6 µl of deionized formamide with 8.3 mM EDTA, pH 8.0. Insoluble materials were removed by brief centrifugation. The DNAs in the supernatant were denatured by heating at 90°C for 2 min and loaded immediately on a pre-electrophoresed 6% polyacrylamide gel with 8 M urea assembled in ABI DNA sequencer, Model 373A (operated by Dr N Shirley of the Department of Plant Science, WARI).

2.2.12 Sequence analysis and determination of secondary structure

The nucleotide sequences of the viroid forms were examined and aligned with $CCCVd_{246}$ for comparison using the Seq. Ed. computer program provided by ABI. The secondary structures representing the lowest free energy were determined by the program developed by Zuker (1989).

CHAPTER 3

DETECTION OF CCCVd-LIKE SEQUENCES IN PLANTS OTHER THAN PALM SPECIES

INTRODUCTION

The natural mode of spread of cadang-cadang is still unknown and several aspects of disease epidemiology still have to be investigated (Randles *et al.*, 1992). One important question is whether alternative plant reservoirs of the viroid occur in the field. Natural and experimental transmission of the viroid has been observed only in palm species (Randles *et al.*, 1980; Imperial *et al.*, 1985). Recently, preliminary evidence has been obtained suggesting that other plant species are natural hosts of CCCVd (Hanold, Rodriguez and Randles, 1989; Hanold and Randles, 1991).

With the development of sensitive, diagnostic methods specific for CCCVd (Schumacher *et al.*, 1983; Imperial *et al.*, 1985; Hanold and Randles, 1991), the search for alternate hosts was pursued in this study by testing a large number of samples from a range of different plant species that grow naturally in coconut plantations.

EXPERIMENTAL

3.1 COLLECTION OF SAMPLES OF PLANT SPECIES ASSOCIATED WITH COCONUT PLANTATIONS

Sampling of the predominant plant species in coconut plantations was done at 11 separate sites (approximately 2 ha in area) in five provinces of southern Luzon, Philippines (see Appendix C). Of these sites, eight had high cadang-cadang incidence, two had low incidence and one had zero incidence. The aim was to collect, at random, at least five plants per species in each site. Occasionally, however, only one plant per species was obtained, or none at all, due to cultivation of the area under the coconuts to plant cash crops. The collected plants were

transplanted and maintained in a screenhouse for experimental use and observation of symptoms. A sample of each species was dried and pressed for botanical identification at the University of the Philippines at Los Banos. Some of the species were identified according to Moody *et al.* (1984) and Dahlgren *et al.* (1985).

The selection of species was concentrated on monocots, although there was one dicot species (*Urena lobata*) that was included because of its common occurrence in the sites surveyed. Of the approximately 900 samples collected, none showed any specific stunting or discoloration.

3.2 ISOLATION OF LOW MOLECULAR NUCLEIC ACIDS

Four extraction methods were compared for efficient isolation of low molecular weight nucleic acids from the different plant species. These include the methods described in Sections 2.2.1, 2.2.2, 2.2.3 and a method developed by Keese and Symons (1987) using a buffer containing 3% SDS, 10 mM $MgCl_2$ and 0.5 M NaCl. Method 2.2.1 was found to be most effective in removing significant amounts of colored and other materials without significant loss of the fraction containing the low molecular weight nucleic acids. This method was, therefore, used throughout. Moreover, large amounts of tissues (at least 20 g) had to be extracted in order to detect unambiguously the presence of CCCVd-like sequences. Thus, in most cases, the whole plant was extracted.

3.3 MOLECULAR HYBRIDIZATION ANALYSES FOR CCCVd

The plant extracts were analyzed for the presence of CCCVd-like sequences by molecular hybridization techniques using either ^{32}P-cRNA (Section 2.1.2) or ^{32}P-cDNA probe (Section 2.2.6a) and partially purified CCCVd as a standard. These probes are composed of single-stranded linear CCCVd RNAs or cDNAs of (-) polarity and therefore, could detect only the infectious (+ sense) CCCVd molecules. The antisense RNA intermediates of the viroid replication cycle which are usually present *in vivo* but in low concentration would not be

detected. The use of a cRNA probe has the advantage in that RNA:RNA hybrids are significantly more stable than DNA:RNA hybrids (McInnes and Symons, 1989).

For routine testing involving a large number of samples, the dot-blot procedure (Section 2.2.6b and c) was used. Selected samples positive by dot-blot assay were further analyzed by electroblot hybridization (Section 2.2.6b and c) to determine the approximate sizes of the nucleic acids hybridizing to the probe.

3.3.1 Dot-blot hybridization assays

Of the 21 plant species screened, 11 species (Fig. 3.1) that were common in each or all of the sites surveyed gave positive results in the dot-blot assays. Five unidentified grass species also gave positive results but were not commonly found in the sites with the result that not enough materials were gathered for further analyses.

A total of 765 samples were collected from the 11 plant species (Table 3.1). *B. blumeana* had the highest number of positives out of the samples assayed (70.4%) while *U. lobata* had the least positives (4.8%). The positive plants came from sites with high, low and zero levels of cadang-cadang in the coconuts. Thus, it is possible that these infected plant species could act as sources of inoculum for coconut palms in the field.

In Fig. 3.2, autoradiograms A and B (at low and high stringency wash, respectively) show that the CCCVd probe bound strongly to some samples but was lost when a high stringency wash was done suggesting that the nucleic acids had low sequence homology with CCCVd. However, some samples gave very strong signals even after a high stringency wash as shown in blots B and C indicating that these samples contained nucleic acids with high sequence homology to CCCVd. The samples assayed in blots B and C came from sites with high and zero cadang-cadang incidence, respectively. This is an example of the observation that CCCVd-like sequences could be detected in some plants regardless of the presence of cadang-cadang infected palms in the vicinity. In the area with zero incidence of cadang-cadang, transmission of these CCCVd-like sequences from the understorey plants to the coconut palms is possible.

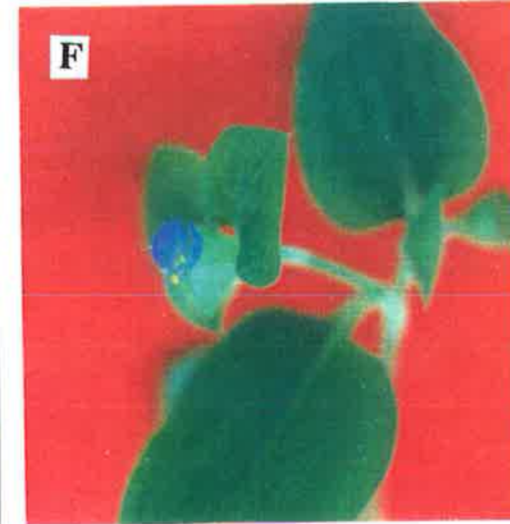
Fig. 3.1 The plant species collected from coconut plantations at 11 sites in southern Luzon, Philippines, that were shown to contain CCCVd-related sequences. Members of the superorder Zingiberiflorae are *Maranta arundinacea* (A), *Alpinia* sp. (B), *Zingiber officinale* (C) and *Canna flaccida* (D). Members of the superorder Commeliniflorae are *Commelina diffusa* (E), *Commelina benghalensis* (F), *Paspalum conjugatum* (G), *Brachiaria distachya* (H), *Imperata cylindrica* (I) and *Bambusa blumeana* (J). *Urena lobata* (K), the only dicotyledon species, is a member of the family Malvaceae whereas the rest belong to five taxonomic families of monocotyledons (refer to Table 1).

A

B

C

D

E

F

G

H

I

J

K

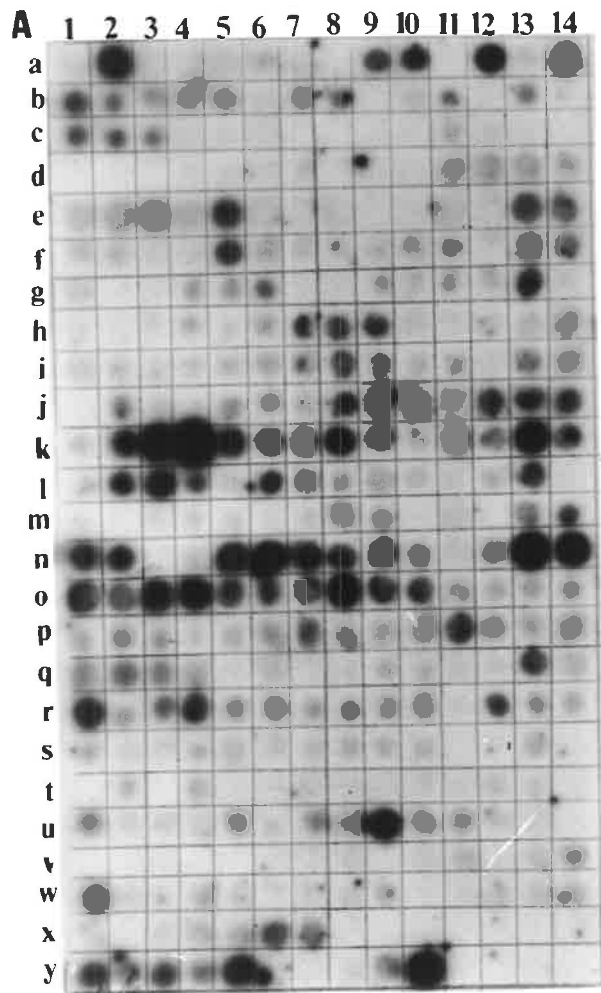
Table 3.1 **Results of molecular hybridization analyses for CCCVd-related RNAs in samples of eleven species from eleven sites in Southern Luzon, Philippines, classified by level of cadang-cadang disease**

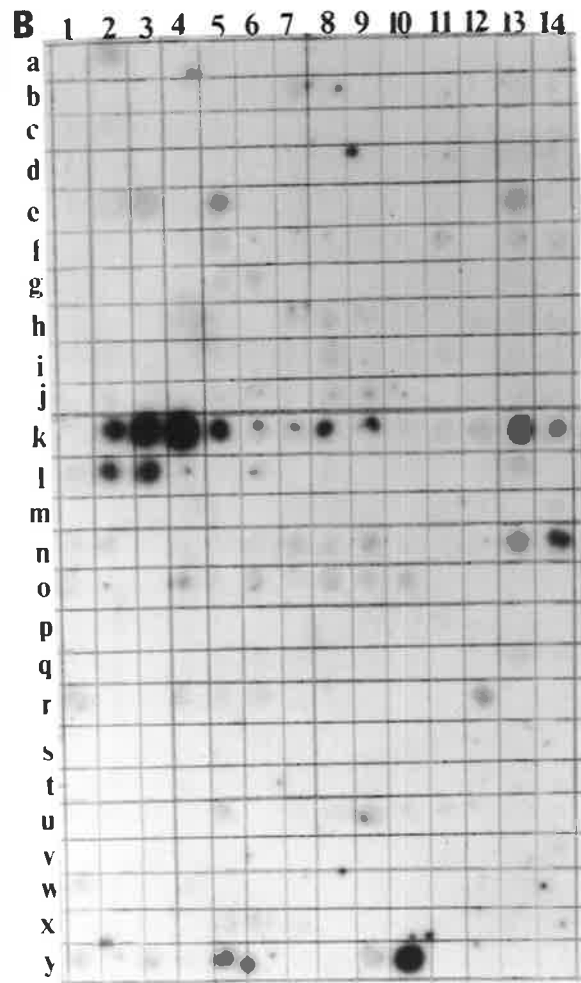
Family	Species	Incidence of positive plants by dot-blot assay[a,b] at sites of:												Electro-blot[a,b,c]
		High incidence								Low incidence		No disease	Total (%)	
		A	B	C	D	E	F	G	H	I	J	K		
Marantaceae	*Maranta arundinacea*	5/39	4/9	30/82	12/30	3/11	9/14	4/10	16/40	0/2	2/11	6/17	91/265 (34.3)	22/50
Zingiberaceae	*Alpinia* sp.	6/17	-	-	1/5	3/6	5/7	0/5	3/9	2/5	1/12	2/5	23/71 (32.4)	8/11
	Zingiber officinale	0/1	0/1	-	-	-	4/5	1/6	1/3	-	-	3/16	9/32 (28.1)	4/7
Cannaceae	*Canna flaccida*	1/5	0/8	3/5	0/5	3/7	1/5	1/5	3/6	0/3	4/7	0/5	16/61 (26.2)	2/4
Commelinaceae	*Commelina diffusa*	5/14	1/2	5/10	2/4	4/5	4/5	4/5	3/6	5/5	2/5	0/5	35/66 (53.0)	2/6
	Commelina benghalensis	8/12	3/5	1/3	-	5/5	4/5	-	3/4	-	0/2	2/6	26/42 (61.9)	4/5
Poaceae	*Paspalum conjugatum*	4/8	2/5	1/5	0/3	0/5	0/5	2/5	1/5	-	1/5	3/10	14/56 (25.0)	4/5
	Brachiaria distachya	1/5	3/4	2/5	-	1/4	4/5	-	-	0/4	1/5	5/6	17/38 (44.7)	4/5
	Imperata cylindrica	3/5	0/5	2/5	4/5	1/2	2/7	0/5	1/2	0/3	-	1/5	14/44 (31.8)	2/4
	Bambusa blumeana	2/2	2/2	2/2	2/3	2/2	3/5	1/1	1/1	1/1	1/5	2/3	19/27 (70.4)	3/5
Malvaceae	*Urena lobata*	1/12	0/4	0/7	0/5	0/6	0/7	0/5	0/3	-	0/7	2/7	3/63 (4.8)	3/4

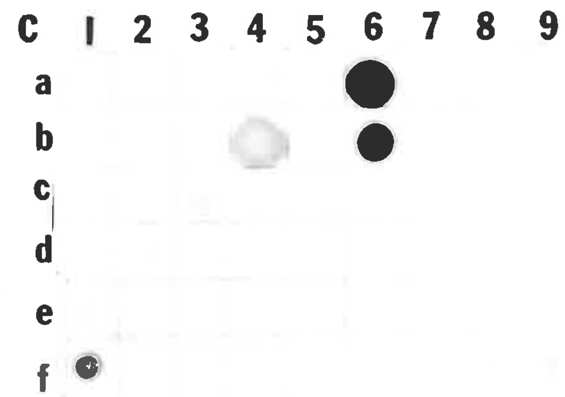
a Ratios are no. of plants positive/no. tested.
b At high stringency wash.
c Samples selected from both positive and negative dot-blots.

Fig. 3.2 Dot-blot assays using the ^{32}P-cDNA probe. A filter with samples from high cadang-cadang incidence areas was probed, washed at low stringency and autoradiographed (A), then re-washed at high stringency and again autoradiographed (B). *M. arundinacea* samples are rows (a-e); *C. benghalemsis*, row (f); *C. diffusa*, rows (g-i); *Alpinia* sp., rows (j-k); *Z. officinale*, row (l); *U. lobata*, row (m); *C. flaccida*, rows (n-o); *B. blumeana*, row (p); unidentified grasses, row (q); *B. distachya*, row (n); *P. conjugatum*, rows (s-t); *I. cylindrica*, row (u) and unidentified grasses, rows (v-y). The CCCVd markers are (y-9) and (y-10). In autoradiogram (C), the filter was washed at high-stringency and the samples were collected from a site with zero level of cadang-cadang disease. The strong signals are *Alpinia* sp. (a-6), *B. distachya* (b-6), *M. arundinacea* (b-4) and the CCCVd marker (f-1).

Each dot is from a different plant.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
a b c d e f g h i j k l m n o p q r s t u v w x y


B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
a b c d e f g h i j k l m n o p q r s t u v w x y


C
1 2 3 4 5 6 7 8 9
a b c d e f

3.3.2 Electroblot hybridization assays

3.3.2a 20% non-denaturing PAGE

Not all of the dot-blot positives were assayed by electroblot (see Table 3.1) because some of the positive plants died when transplanted and before sufficient amounts of tissues were collected. Moreover, some samples became negative when re-assayed by dot-blot and this could have been due to degradation of the CCCVd-like nucleic acids during storage or transit of the samples.

The electroblot results after a high stringency wash are summarized in Table 3.2 and the autoradiograms of some selected samples from each of the 11 species are shown in Fig. 3.3B and Fig. 3.4. All of the species, regardless of the site they came from, had bands within the monomer region of CCCVd that ranges in size from 246 to 297 nts. Bands were also detected in the dimer region as well as bands between the monomers and the dimers.

The approximate sizes of the other nucleic acids present in the plant species which hybridized to the CCCVd probe only at a low stringency wash were determined by comparing autoradiograms A and B (at low and high stringency wash, respectively) in Fig. 3.3. The nucleic acids with low homology with CCCVd were generally not of the same size as CCCVd.

3.3.2b Two-dimensional 7% PAGE

To show the circularity of the detected CCCVd-like nucleic acids in the plant species, the samples were fractionated on 2-D non-denaturing/denaturing 7% PAGE (Section 2.2.4a). Then, the nucleic acids were electroblotted on nylon membrane and hybridized with CCCVd probe (Section 2.2.6b and c). An example of this assay is shown in Fig. 3.5 where two circular molecules of about the same size as the CCCVd monomers and a molecule of about the size of one of the CCCVd dimers were detected in *M. arundinacea*. Two-dimensional gel electrophoresis analysis of isolates of the other species are presented in Chapter 5 where the circular bands were visualized by ethidium bromide staining (Section 5.1.1).

Table 3.2 **Approximate sizes (nts) of CCCVd-related RNAs detected in various species from sites with zero, low and high incidence of cadang-cadang**

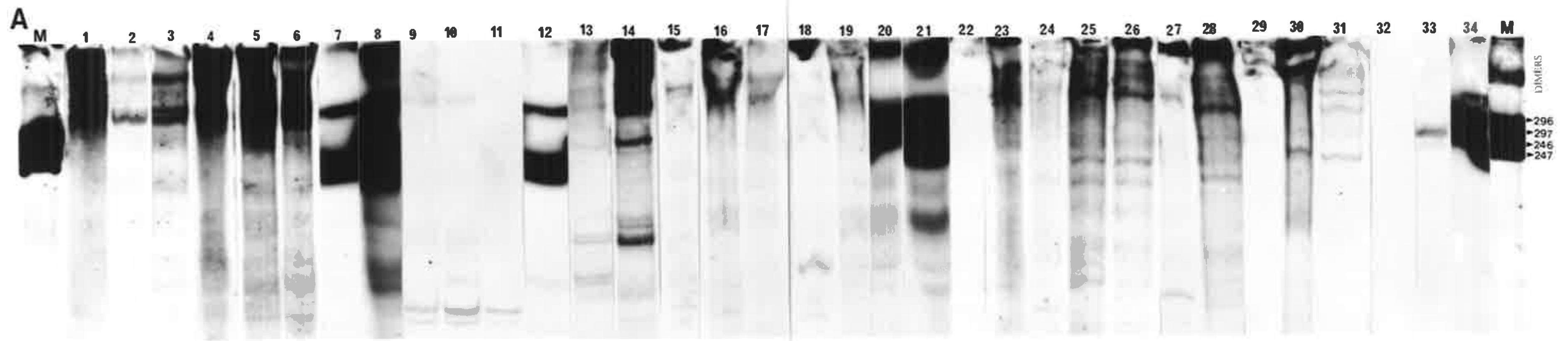
Species	No. of samples	Band patterns relative to marker CCCVd in 20% non-denaturing PAGE-electroblot*						Example in Fig.**	Incidence of cadang-cadang where sample in Fig. was collected
		247	246	297	296	<Dimer >296	Dimer		
M. arundinacea	11	+					+	4B#3	High
	6	+	+			+		3B#7	Zero
	5					+	+	3B#5	Low
Alpinia sp.	3	+						4A#4	High
	1	+			+			4A#5	High
	3	+	+			+		3B#12	Zero
	1	+	+		+			3B#11	High
Z. officinale	2	+	+					4B#1	High
	2	+					+	4B#2	High
C. flaccida	2	+	+					3B#14	Zero
C. diffusa	2	+						4B#5	High
C. benghalensis	2	+					+	4B#8	High
	1	***	***	***	***			3B#21	Zero
	1					+		3B#20	Zero
P. conjugatum	4	+					+	4B#7	High
B. distachya	4	+	+				+	4B#6	High
I. cylindrica	2	+	+				+	4B#12	High
B. blumeana	2		+					4B#10	High
U. lobata	1		+					4B#9	High
	1		+	+				3B#33	Zero
	1					+		3B#34	Zero

* After high stringency wash.

** This Chapter.

*** Specific size of monomer not determined (whether band was of 247, 246, 296 or 296 nts) due to high signal strength.

Fig. 3.3 Hybridization assay of electroblots from 20% non-denaturing PAGE using ^{32}P-cDNA probe. Electroblots were washed first at low stringency, exposed (A), then re-washed at high-stringency before exposure (B). *M. arundinacea* samples are lanes (1-8); *Alpinia* sp., lanes (9-12); *C. flaccida*, lanes (13-14); *C. diffusa*, lanes (15-17); *C. benghalensis*, lanes (18-21); *P. conjugatum*, lanes (22-23); *B. distachya*, lanes (24-26); *I. cylindrica*, lanes (27-28); *B. blumeana*, lanes (29-31) and *U. lobata*, lanes (32-34). M denotes CCCVd marker.


A
M
1
2
3
4
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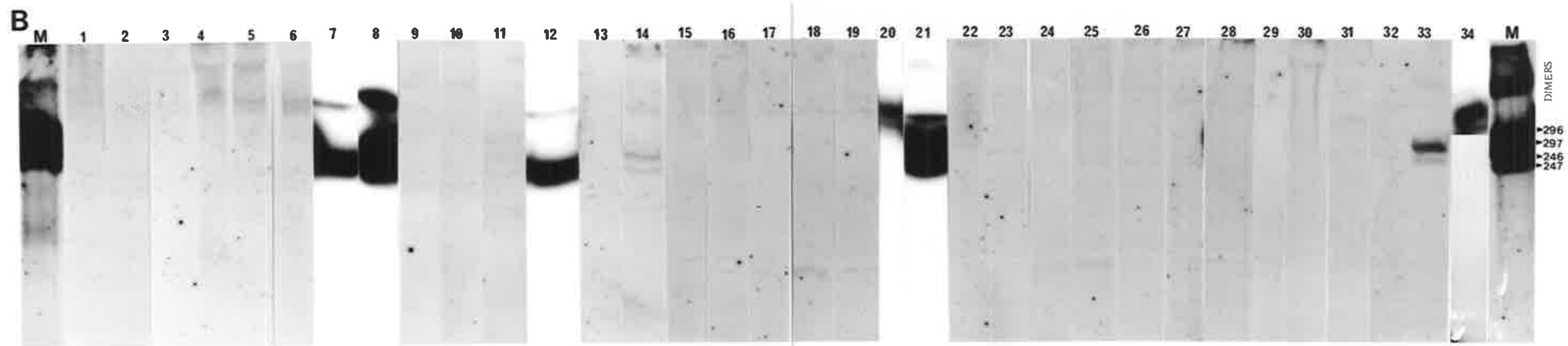
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
M
DIMERS
296
297
246
247


B
M
1
2
3
4
5
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7
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11
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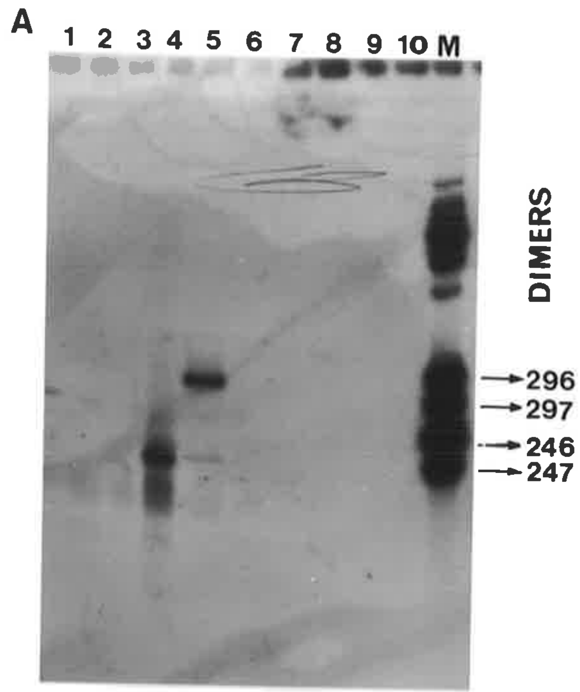
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
M
DIMERS
296
297
246
247

Fig. 3.4 High stringency wash hybridization assay of electroblots from 20% non-denaturing PAGE using ^{32}P-cRNA probe. In autoradiogram (A), lanes (3-5) are *Alpinia* sp. samples. In autoradiogram (B), *Z. officinale* samples are lanes (1-2); *M. arundinacea*, lanes (3-4); *C. diffusa*, lane (5); *B. distachya*, lane (6); *P. conjugatum*, lane (7); *C. benghalensis*, lane (8); *U. lobata*, lane (9); *B. blumeana*, lane (10); *Alpinia* sp., lane (11) and *I. cylindrica*, lane (12). M denotes CCCVd marker.


A
1 2 3 4 5 6 7 8 9 10 M
DIMERS
296
297
246
247

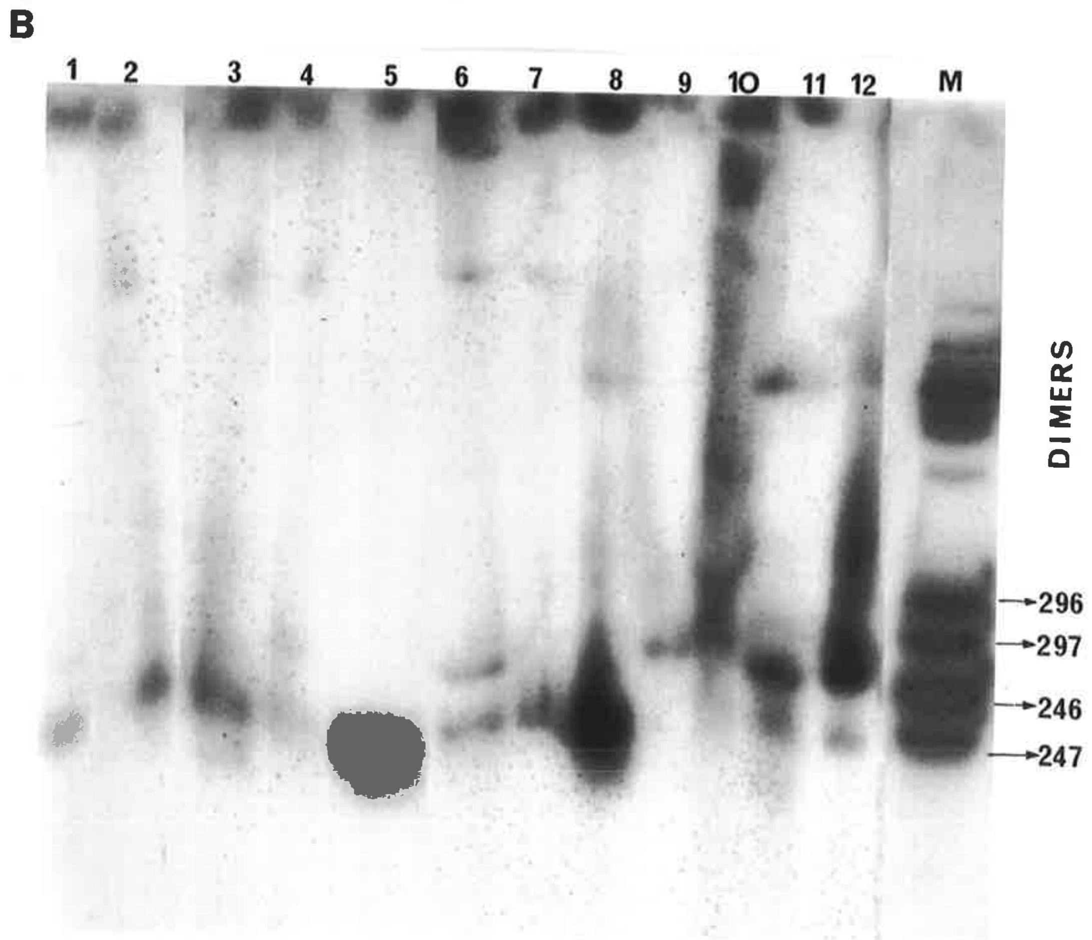
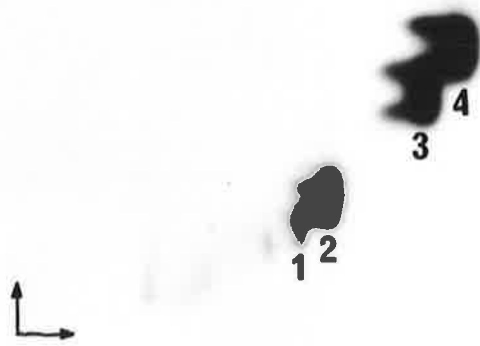

B
1 2 3 4 5 6 7 8 9 10 11 12 M
DIMERS
296
297
246
247

Fig. 3.5 High stringency wash hybridization assay of electroblots from 2-D 7% PAGE using ^{32}P-cDNA probe. In autoradiogram (A), the circular CCCVd molecules from a partially purified extract are numbered in the order of increasing electrophoretic mobility. Band no. (4) is the small monomer (246/247 nts); no. (3), the large monomer (296/297 nts) and nos. (2) and (1) are their corresponding dimers. In autoradiogram (B), the circular molecules from a partially purified extract of *M. arundinacea* are also numbered in the same way as those of CCCVd marker. Bands, nos. (3) and (2), are about the size of the small and large CCCVd monomer, respectively, while band no. (1) corresponds to the size of one of the CCCVd dimers.

A



B

DISCUSSION

CCCVd-like sequences have been detected in 11 plant species commonly found in coconut plantations. One of these species is a dicot and the rest belong to five families of monocotyledons from the superorders Commeliniflorae and Zingiberiflorae. As CCCVd-related sequences could be detected in these plant species regardless of the incidence of cadang-cadang in coconuts, the infected understorey plants could act as sources of inoculum for coconuts in the field.

Of the four extraction methods tested, the most effective procedure was blending of the plant tissues with 0.5 M sodium acetate, 10 mM $MgCl_2$, 20% ethanol, 3% SDS and an equal volume of water-saturated phenol followed by a series of phenol/chloroform extractions and CTAB/LiCl purifications.

The CCCVd-like nucleic acids were found to occur in very low concentrations in the plant species. Thus, large amounts of tissues (at least 20 g) had to be extracted for these nucleic acids to be detected by the diagnostic methods described in this study. No apparent symptom could be observed in the positive plants suggesting that the infection was latent.

There is some degree of sequence and size heterogeneity between these CCCVd-like nucleic acids and CCCVd, thus, nucleic sequencing is necessary to compare the different isolates and to determine their degree of relatedness to CCCVd. Since they are present in low concentrations in the plant tissues, amplification by the polymerase chain reaction (PCR) is necessary to provide sufficient amount of nucleic acids either for direct sequencing or for molecular cloning. It is often desirable, however, to construct clones so that diagnostic probes can be made for subsequent studies such as viroid replication and epidemiology. Furthermore, the infectivity of these CCCVd-like nucleic acids can be tested using the clones.

CHAPTER 4

DETECTION OF ELECTROPHORETIC FORMS OF CCCVd ASSOCIATED WITH SEVERE BROOMING SYMPTOMS IN COCONUT

INTRODUCTION

The naturally occurring sequence variants of CCCVd reported so far are discussed in Section 1.2.3. Variants which arise during disease progression all contain the basic 246 nucleotides.

The difference between variants 246 and 247 as well as 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). Using this procedure for routine diagnosis of CCCVd in coconut palms, minor bands which have not been described previously were observed in positions between the four monomeric CCCVd forms in some cadang-cadang infected palms. In this chapter, the relationship between the presence of these bands and the severity of symptoms of the disease was investigated.

EXPERIMENTAL

4.1 OBSERVATION OF SEVERE "BROOMING" SYMPTOMS IN CCCVd INOCULATED PALMS

At the Albay Research Center (ARC) of the Philippine Coconut Authority (PCA), Philippines, inoculation of various coconut populations with CCCVd preparation is done with a hand-primed high pressure injector to seek resistance or tolerance to viroid infections. 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 materials obtained from various palms in local coconut plantations or at the ARC. Preparations of inoculum are stored in ethanol at -20°C which remain infective for at least three months (Randles *et al.*, 1992). Approximately 1 ug of CCCVd is inoculated into each test palm.

For the early inoculations shown in Table 4.1, 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 post emergence rather than when the sprouts were 2-3 mo-old. In addition, the rate of symptom development varied (not shown in Table 4.1). An obvious new symptom type appeared in a significant number of palms. It was characterized mainly by loss of leaf lamina, giving the palm a "broomed" appearance associated with severe stunting of the palm (Fig. 4.1). 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 4.1). In the 2-3 mo-old inoculated palms, the brooming symptom appeared 4-7 years after inoculation while in the 1-7 d-old 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% to 12.9%). Premature death was observed in some of the palms. Although there is no direct evidence that this was caused by the severity of the disease, this observation is unusual even for palms with the typical cadang-cadang symptoms.

Table 4.2 shows the results of an infectivity trial where the variants of CCCVd determined by their mobility in the gel were isolated from each other and tested for their ability to infect. Ten-fold dilution of each variant was done starting from 200 ng/test palm to 200 pg/test 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. Furthermore, brooming incidence was essentially confined to palms inoculated with the 246 or 247 variant, with only one case of a brooming palm occurring among those inoculated with the 296 variant.

In all the trials conducted, a total of 1,787 successfully inoculated palms have been monitored and 12% of these had brooming symptoms.

Table 4.1 Incidence of the brooming symptom in various coconut populations inoculated with CCVd 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	
		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) x Baybay	6	11/30	-	0/11	
		Catigan x Rennel	6	5/30	6	3/5	
		Tacunan x West African Tall (WAT)	6	8/30	6	1/8	18.8
1982	2 mo	Malayan Yellow Dwarf (MYD)	5	1/17	-	0/1	
		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	22.7
1983	2 mo	mixed populations	4	300/750	4	18/300	
		MYD x WAT	4	3/24	-	0/3	
		OD x WAT	4	3/24	-	0/3	5.9

Table 4.1 (Continued)

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
1983	1-7 d	MYD x WAT	4	11/24	-	0/11	
		STB** x STP**	4	14/24	5	4/14	
		CRD x CRD	4	15/22	5	2/15	
		STP x STP	4	21/24	5	5/21	
		YD** x LT**	4	21/24	5	4/21	
		OD x WAT	4	15/24	-	0/15	
		STB x STB	4	18/24	-	0/18	
		CRD x LT	4	13/23	5	1/13	
		OD x LT	4	19/24	5	3/19	12.9
1985	1-7 d	Tambolilid	2	286/550	3	17/286	
		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	4.4
1986	1-7 d	Tambolilid	1	242/450	4	17/242	7.0

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

Fig. 4.1 The unusual, severe brooming symptoms observed in coconuts inoculated with CCCVd variants 246 and 247.

A. The palm is severely stunted with a "broomed" appearance due to the reduction of the lamina. The basal fibres remain attached to the fronds as in a typical cadang-cadang infected palm.

B. The frond is shorter (left) compared to a healthy frond (right).

C. Leaflets have reduced laminae (left) as compared to cadang-cadang infected leaflets (middle) and healthy leaflets (right).

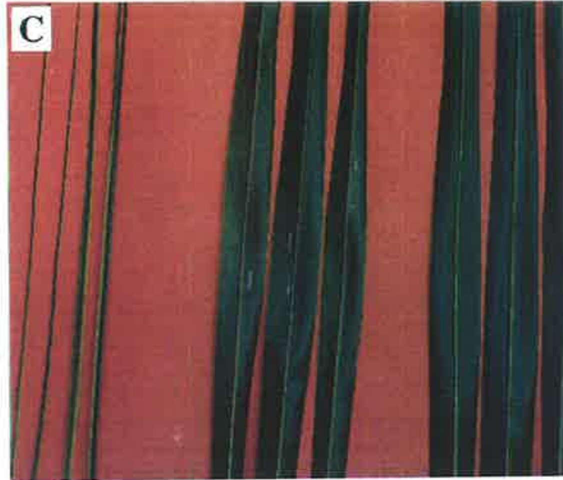


Table 4.2 Incidence of the brooming symptom in Tambolilid palms inoculated 1-7 d post emergence with purified CCCVd

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

4.2 CORRELATION OF THE BROOMING SYMPTOMS WITH DISTINCT ELECTROPHORETIC FORMS OF CCCVd

The PAGE assay of palms with brooming symptoms was done as described in Sections 2.2.2 and 2.2.5. 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 a palm since the first detection of the brooming symptoms. This was followed by another whole-crown assay approximately one 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 and the viroid patterns obtained are summarized in Table 4.3. 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 and 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; two, between 246 and 297; three, between 297 and 296 and three, above 296.

Fig. 4.2 shows examples of viroid patterns from palms with varying intensities of the brooming symptoms. The distinct forms fractionated in 20% non-denaturing PAGE were visualized by silver nitrate staining (Fig. 4.2A) as described in Section 2.2.5 and by hybridization to a ^{32}P-labelled cDNA probe (Fig. 4.2B) as described in Section 2.2.6. Their circularity was confirmed by 2-D PAGE (Section 2.2.4a) in which a 20% non-denaturing gel was used in the first dimension and a 10% denaturing gel was used in the second dimension (Fig. 4.2C). These viroid forms could not be detected in 5% and 10% non-denaturing PAGE suggesting that there were only minor differences in their nucleotide sequences compared with the common CCCVd forms. These results indicate that the electrophoretic forms associated

Table 4.3 **Viroid patterns in 20% non-denaturing PAGE* of palms with the brooming symptom shown in relation to frequency of detection**

MONOMER REGION**

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

Pattern no.	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47
296c					•								•						•	•		•	
296b																			•			•	
296a				•					•		•	•			•				•		•		
296		•	•			•												•	•		•	•	
297c									•														
297b	•	•					•			•	•			•	•	•	•	•	•	•			
297a							•																•
297						•		•								•			•				
246b																							
246a																							
246														•									
247c								•	•	•	•	•	•										
247b	•	•	•	•	•	•	•							•	•								
247a								•	•	•	•	•	•										
247																							
No. of palms screened	1	2	4	2	3	1	1	2	2	2	2	2	5	1	3	3	8	3	1	1	1	1	2

Total = 136

* Whole-crown assay

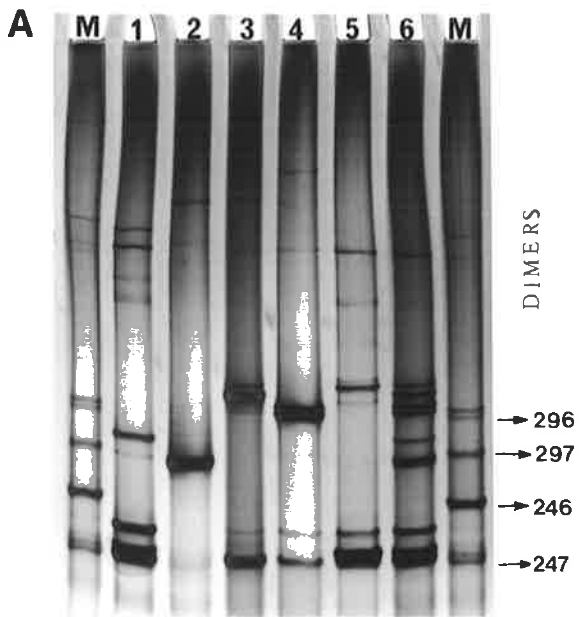
** Positions of the electrophoretic forms designated with small letters are shown relative to the marker $CCCVd_{247,246,297,296}$.

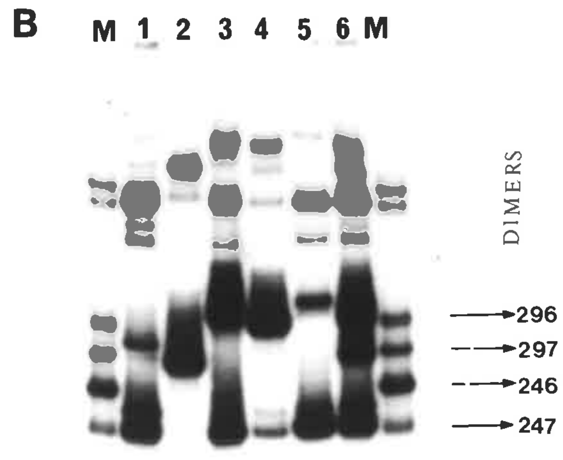
Fig. 4.2 Assay of some palms with varying intensities of the brooming symptoms.

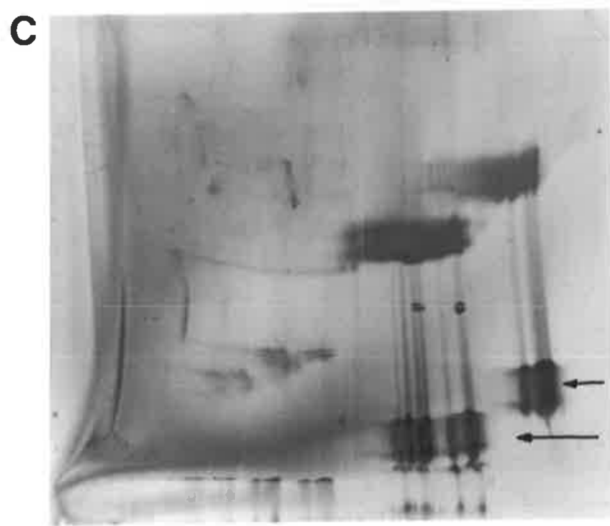
A. 20% non-denaturing PAGE assay of extracts from palms, nos. 1-5 (see Table 4.4) and a mixture of their isolates in lane no. 6. The bands were visualized by silver nitrate staining.

B. Hybridization assay of an electroblot from a 20% non-denaturing PAGE of the samples in A above.

C. Two-dimensional PAGE of a mixed isolate (lane no. 6 in A and B) with 20% non-denaturing gel in the first dimension and 10% denaturing gel in the second dimension. The arrows show the circular bands.


A
M
1
2
3
4
5
6
M
DIMERS
296
297
246
247


B
M
1
2
3
4
5
6
M
DIMERS
296
297
246
247


C

with the severe brooming symptoms differed in primary and secondary structure from CCCVd, and are probably mutants of CCCVd.

4.3 VARIATION IN THE VIROID PATTERNS DURING DISEASE DEVELOPMENT

Twelve palms were analyzed for their viroid patterns from the time the viroids were first detected until the brooming symptom appeared (Table 4.4). The final PAGE assays of five of these palms are shown in Fig. 4.2. It was observed that 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 with the size of the molecules increasing. However, there were at least two palms (with viroid patterns 2 and 3, Table 4.3) 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.

DISCUSSION

A severe "brooming" symptom was observed in approximately 12% of the CCCVd-inoculated palms screened. The symptom is characterized by the loss of leaf lamina, hence, the brooming appearance associated with severe stunting of the palm, and occasional premature death. Observed 3-7 years after inoculation, it was essentially confined to palms inoculated with either 246 or 247 variants of CCCVd.

PAGE and hybridization analyses have shown that all brooming palms have distinct electrophoretic forms of CCCVd. The next step is to sequence these forms to determine whether the severity of the disease is caused by mutations in CCCVd.

Table 4.4 Time-course analysis of viroid pattern prior to the appearance of the brooming symptom

Palm sample	Year inoc-ulated	Year brooming was observed	Viroid pattern 1984	1985	1986	1987	1988	1989	1990	Pattern no. in Table 4.3	Isolate no. in Fig. 4.2
Magtuod 1	1982	1987	247	NA	247, 247a	247, 247a, 247b, 297, 297a				17	1
STB x STP 1	1983	1988	NA*	247	247	247, 246b	247, 246b			9	2
OD x LT 1	1983	1988	247	NA	247	247, 247b	247, 247b, 296, 296a, 296b			16	3
STP x STP 1	1983	1988	-	NA	247, 246	NA	247, 247b, 297c, 296			14	4
STP x 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	

* Not assayed.

** Positive in dot-blot assay.

CHAPTER 5

PURIFICATION OF CCCVd-RELATED NUCLEIC ACIDS FOR AMPLIFICATION BY POLYMERASE CHAIN REACTION (PCR) AND THEIR SUBSEQUENT MOLECULAR CLONING

INTRODUCTION

A commonly used approach for purification of viroids has been to prepare a low molecular weight nucleic acid extract of infected plant tissue and then to separate the circular viroids from the contaminating cellular linear RNAs (Schumacher *et al.*, 1983; Rivera-Bustamante *et al.*, 1986). In a denatured state, the circular viroids have much lower electrophoretic mobility than linear RNAs of the same size (Schumacher *et al.*, 1983). Viroids isolated in this way have proved to be pure enough for many purposes such as direct RNA sequencing or *in vitro* synthesis of viroid cDNA for cloning (Keese and Symons, 1987).

Some viroids are present in plant tissues in small amounts so that their isolation, purification and subsequent molecular analysis is difficult. However, with the introduction of the polymerase chain reaction (PCR) technology, viroid amplification via its cDNA overcomes this problem (Puchta and Sanger, 1989). The PCR is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA (White *et al.*, 1989). The thermostable DNA polymerase isolated from *Thermus aquaticus* (*Taq* polymerase) (Chien *et al.*, 1976), has allowed simplification of the procedure by enabling the amplification reaction to be performed at higher temperatures. This significantly improves the speed, specificity, yield and sensitivity of the procedure as well as the length of products that can be amplified (Saiki *et al.*, 1988). A three-step cycling process is involved: (1) denaturation of double-stranded (ds) DNA; (2) annealing of primers to complementary sequences of each single-stranded (ss) DNA template; and (3) primer extension by *Taq* DNA polymerase along both template strands in opposite, overlapping directions. This can lead to the many million-fold amplification of the target DNA fragment over the course of 20 to 30 cycles (White *et al.*, 1989). However, optimization of the PCR technique is somewhat empirical. Individual reaction components and time/temperature

parameters must be adjusted for efficient amplification of specific targets. The optimization and interaction of these factors and general guidelines for achieving the best results are discussed by several authors (Williams, 1989; Sambrook *et al.*, 1989; Kwok and Higuchi, 1989; Krawetz *et al.*, 1989; Sarkar and Sommer, 1990; Lowe *et al.*, 1990; Ruano *et al.*, 1991; Ausubel *et al.*, 1991).

For certain applications PCR can obviate the need for cloning, as amplification products can be analyzed directly. However, many strategies generate complex mixtures of products, necessitating molecular cloning for further analyses of specific target molecules (Mead *et al.*, 1991).

Difficulties are frequently encountered when cloning PCR products. A simple method, however, has been shown to be efficient and is based on the observation that *Taq* polymerase can add a single non template-directed deoxyadenosine (A) residue to the 3' end of duplex PCR products (Clark, 1988). This template-independent activity of *Taq* polymerase has been exploited to create a cloning scheme which has the efficiency of sticky-end cloning, but requires no additional enzymatic modification of the PCR product (Holton and Graham, 1991; Marchuk *et al.*, 1991).

This chapter describes the purification of CCCVd-related nucleic acids for amplification by PCR and molecular cloning of the PCR products.

EXPERIMENTAL

5.1 PURIFICATION

5.1.1 CCCVd-related nucleic acids in plants other than palm species

Preparations of CCCVd-related nucleic acids in plants other than palm species were subjected to 2-D gel electrophoresis (Section 2.2.4a). This method, although laborious as only one or two samples can be analyzed on a single gel, is efficient in determining the gel positions of unknown viroids varying in size and sequence.

Fig. 5.1 shows the results of 2-D gel analyses of partially purified extracts of some of the plant species compared with the pattern for CCCVd. The circular molecules detected in each species were numbered in the order of increasing electrophoretic mobility. Six circular bands were detected in the CCCVd isolate, five in *M. arundinacea*, five in *Alpinia* sp., four in *C. flaccida*, four in *C. diffusa* and three in *B. blumeana*. The zones containing these molecules were separately excised and eluted from the gels as described in Section 2.2.4b.

5.1.2 Electrophoretic forms of CCCVd associated with the brooming symptoms in coconut

Listed in Table 5.1 are the isolates extracted as described in Section 2.2.3 from palms with varying intensities of the brooming symptoms. These partially purified isolates showed viroid patterns that are more or less representative of the total brooming-associated electrophoretic forms detected in the 136 palms screened (Table 4.3). Either singly or mixed, the isolates were purified further by bi-directional PAGE (Section 2.2.4a). With the bi-directional system, large amounts of sample can be applied on a single gel since the position of the electrophoretic forms in the native gel relative to the CCCVd marker is known (an example is shown in Fig. 5.2). The purified isolates were again analyzed for their viroid pattern on 20% non-denaturing PAGE (Fig. 5.3). Since most of the isolates had more than one viroid form, attempts were made to separate each form from the others in a long first dimension non-denaturing gel of the bi-directional PAGE. The separation was not successful particularly for those forms very close to each other as shown in Fig. 5.3, B1 and B2.

5.2 POLYMERASE CHAIN REACTION (PCR)

In this study, CCCVd variant 246 and three sets of CCCVd-specific DNA primers were used to determine the parameters suitable for PCR with CCCVd.

Fig. 5.1 Two-dimensional (2-D) 7% PAGE where a lane with one sample in the first dimension non-denaturing gel was cut vertically and placed horizontally below the second dimension denaturing gel. The samples are: pooled extracts from coconut palms at different stages of the cadang-cadang disease (A); *M. arundinacea* (B); *Alpinia* sp. (C); *C. flaccida* (D); *C. diffusa* (E) and *B. blumeana* (F). The circular molecules which migrate behind the diagonal front of the linear nucleic acids are numbered in the order of increasing electrophoretic mobility. The gels were stained with ethidium bromide.

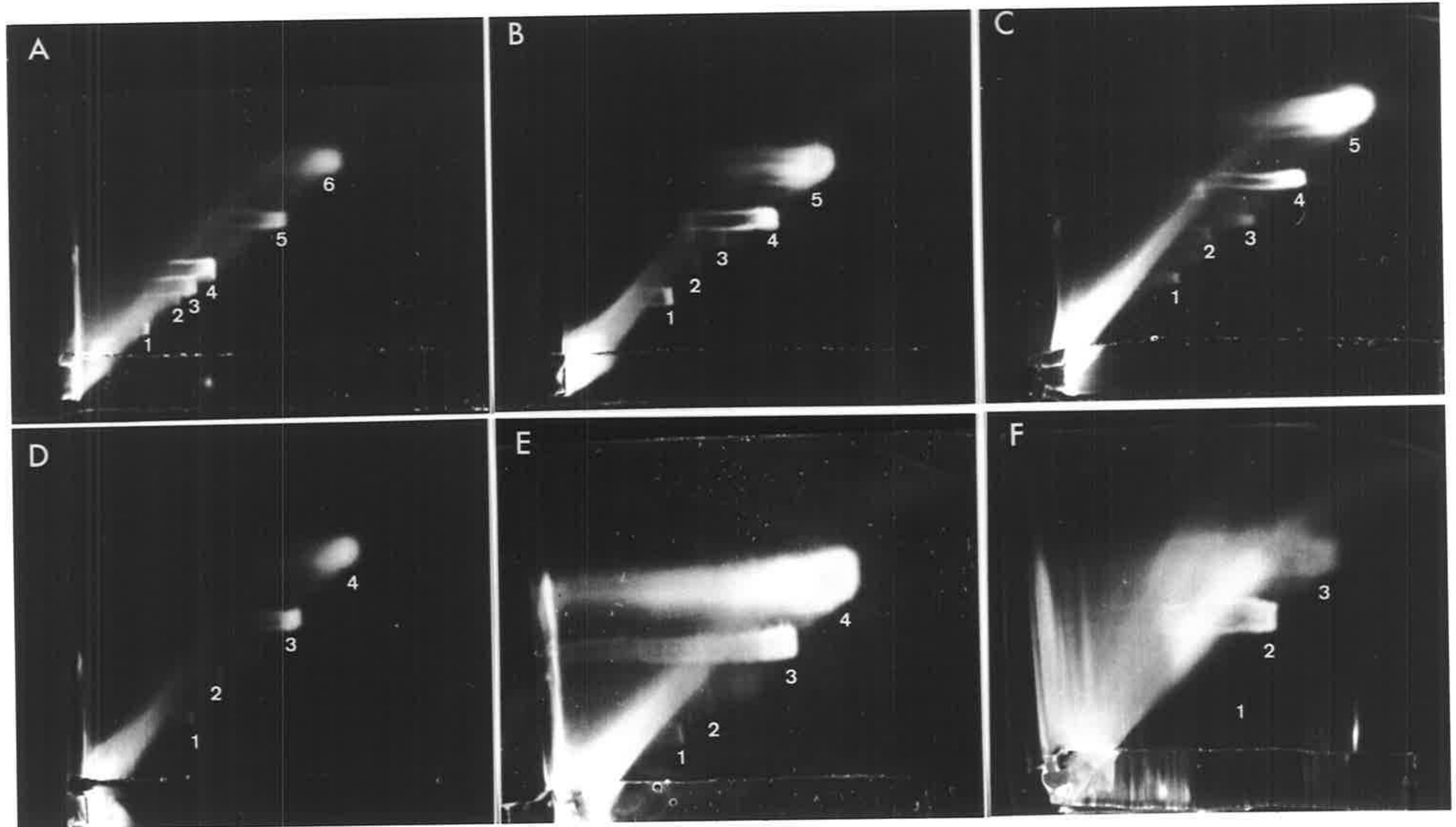

A
6
5
4
3
2
1
B
5
4
3
2
1
C
5
4
3
2
1
D
4
3
2
1
E
4
3
2
1
F
3
2
1

Table 5.1 Partially purified isolates from the different batches of inoculated palms with brooming symptoms and their viroid patterns by electrophoresis in 20% non-denaturing polyacrylamide gels

Isolate	Year isolate was collected	Palm source	Year palm was inoculated	Viroid pattern*
1	1988	Magtuod #1	1982	247, 247a, 247b, 297, 297a
2	1988	STB X STP #1	1983	247, 246b
3	1988	ODX X LT #1	1983	247, 247b, 296, 296a, 296b
4	1988	STP X STP #1	1983	247, 247b, 297c, 296
5	1988	STP X STP #2	1983	247, 247a, 247b, 296, 296b, 296c
6	1988	Tambolilid #1	1985	247b, 246, 246a
7	1989	Tambolilid #2	1985	247a, 247b
8	1989	Tambolilid #3	1985	247a, 247b
9	1989	Tambolilid #4	1985	247b
10	1989	Tambolilid #5	1985	247, 247b
11	1989	Tambolilid #6	1985	247b
12	1990	Tambolilid #7	1986	247, 247b
13	1990	Tambolilid #8	**	297a

* See Table 4.3.

** The only case where the brooming symptom was observed in a palm without severe stunting. Date of inoculation and previous assay were not known, however, it belongs to the earlier batches of inoculated palms (approximately 1981).

Fig. 5.2 Ethidium bromide stained 20% non-denaturing preparative gel (first dimension) of a bi-directional PAGE. Each slot has the same sample which was a mixture of partially purified isolates, nos. 1-6 (Table 5.1). The positions of the electrophoretic forms relative to the CCCVd marker (M) are shown and the gel was cut so that the four bands (forms 247, 246a, 247b and 246) within the 247 and 246 region of the CCCVd marker were separated from approximately nine bands (forms 246a, 246b, 297, 297a, 297c, 296, 296a, 296b and 296c) within the 297 and >296 region. The cut gel slices were each placed at the bottom of a 7% denaturing gel for the second dimension (not shown).

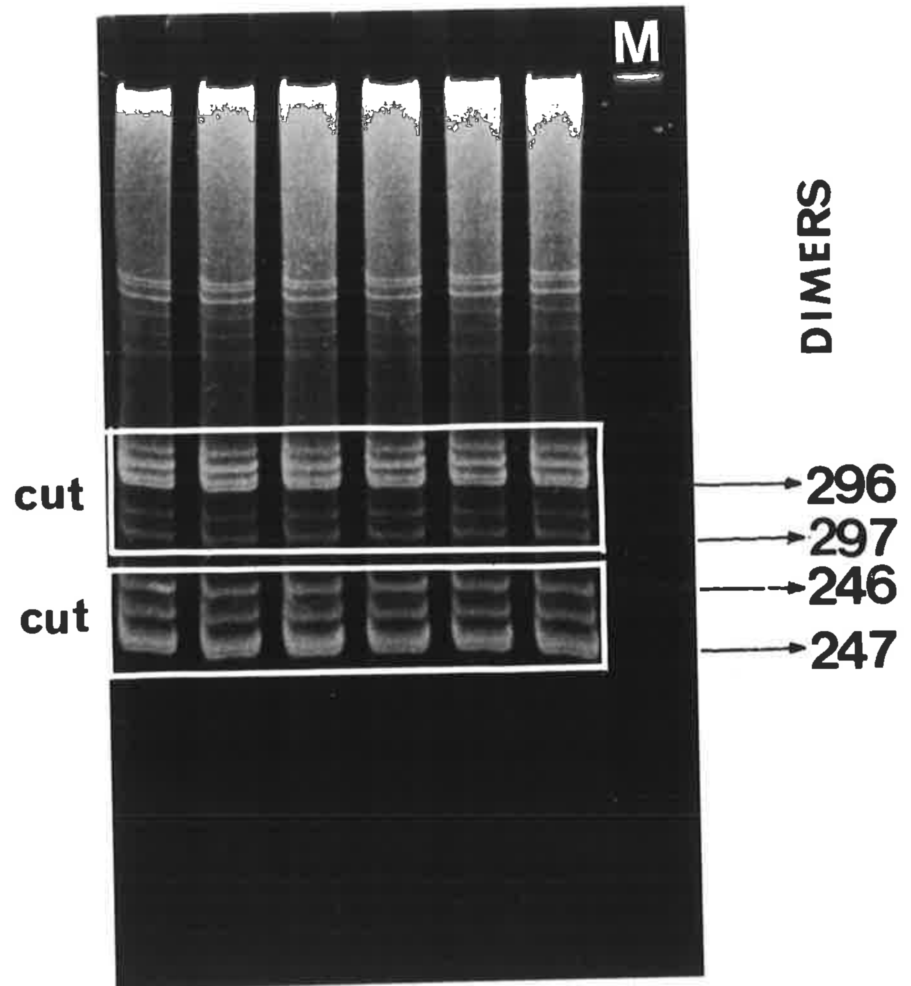

M
DIMERS
cut
cut
296
297
246
247

Fig. 5.3 Analysis on 20% non-denaturing polyacrylamide gels (ethidium bromide stained) of the brooming isolates (Table 5.1) purified by bi-directional PAGE. M denotes CCCVd marker.

A-1 Isolate no. 12 from a single palm with electrophoretic forms 247 and 247b.

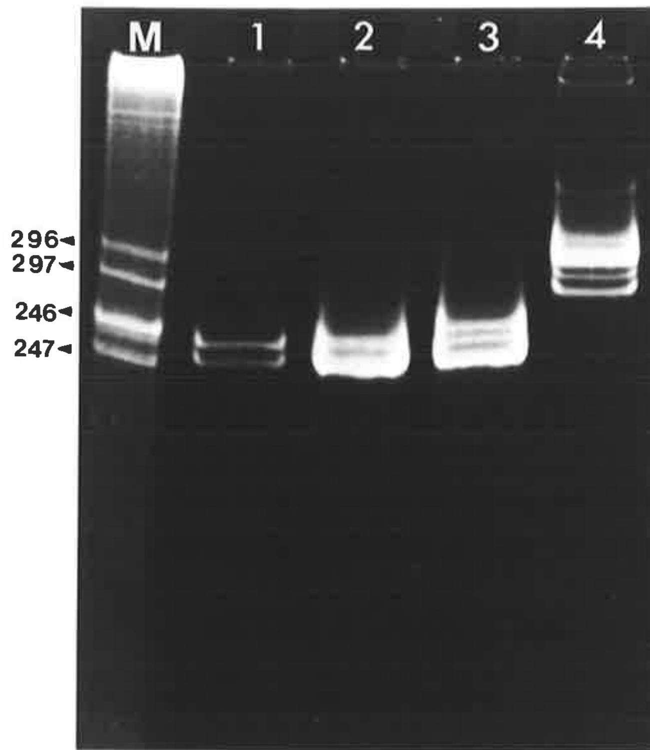
2 Mixed isolate (nos. 7-11) with electrophoretic forms 247, 247a and 247b.

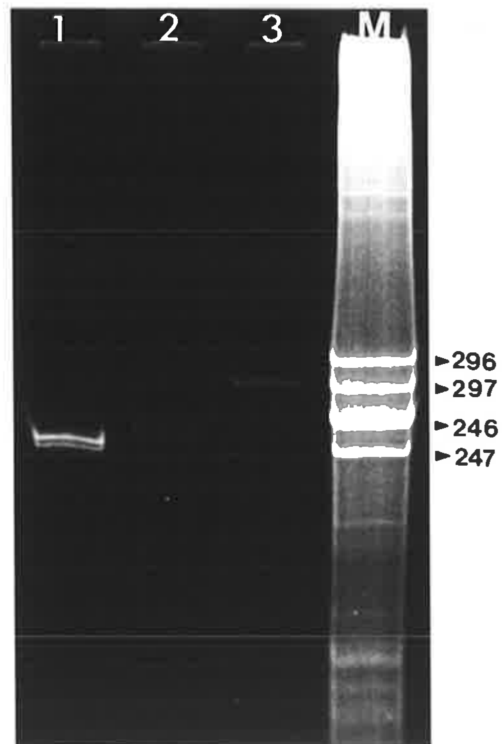
3 Mixed isolate (nos. 1-6) with electrophoretic forms 247, 247a, 247b and 246 (see Fig. 5.2).

4 Mixed isolate (nos. 1-6) with electrophoretic forms 246a, 246b, 297, 297a, 297c, 296, 296a, 296b and 296c (see Fig. 5.2).

B-1 and 2. Separation of the electrophoretic forms in mixed isolate (A-2) above.

3 Isolate no. 13 from a single palm with electrophoretic form 297a.


A
M
1
2
3
4
296
297
246
247


B
1
2
3
M
296
297
246
247

5.2.1 Standardization of PCR

5.2.1a Design of primers and preparation of template

Since the starting material is an RNA, the first factor to consider is the design of a CCCVd-specific DNA primer that will efficiently prime the synthesis of the first strand cDNA. For this cDNA to be amplified by PCR, the same primer is used again while a second primer which is complementary to the 3'-end of the ss cDNA simultaneously primes the second strand synthesis resulting in a ds cDNA as the PCR product.

To carry out the above reactions, three sets of primers (Table 5.2) derived from the sequence of CCCVd variant 246 (Haseloff *et al.,* 1982) were used. With primer sets I and II, the left and right half-lengths of the total CCCVd sequence, respectively, were amplified only when the synthesis of the first strand cDNAs was done using the AMV reverse transcriptase method (Section 2.2.7a). On the other hand, full-length CCCVd was amplified using primer set III and the M-MuLV reverse transcriptase method (Section 2.2.7a). The priming sites for primer sets I and II are at the highly conserved central region of CCCVd where there is more secondary structure and more G-C base pairs than in the priming site for primer set III. Thus, it is concluded that the AMV reverse transcriptase method must have provided the right conditions for the melting of the viroid and annealing of the primer.

5.2.1b $MgCl_2$ concentration

The concentration of $MgCl_2$ in the buffer is a variable that can greatly influence the outcome of PCR. Thus, the optimum concentration of this component has to be determined. Although each component of PCR can affect its outcome, most do not lend themselves as readily to optimization as does $MgCl_2$ concentration (Ausubel *et al.,* 1991).

According to Puchta and Sanger (1989), the $MgCl_2$-concentration for optimal amplification of HSVd cDNA ranges between 0.7-1.0 mM. An experiment was, therefore, done to determine the $MgCl_2$ concentration for optimal amplification of CCCVd. The results showed (Fig. 5.4, A) that there was minimal difference between the rates of amplification for

Table 5.2 **Properties of the DNA primers used for the reverse transcription (RT) and polymerase chain reaction (PCR) of $CCCVd_{246}$**

CCCVd-specific DNA primer					
Primer Set	**Sequence**	**Polarity[a]**	**Binding site at $CCCVd_{246}$[b] (nucleotides 5'→3')**	**Reaction**	**Amplified CCCVd cDNA fragment[b,c] (product length)**
I	5'-d (GGTTTCCCCGGGGATCCCTC)-3'	-	70-51	RT + PCR	50-1/246-195 (102 nts)
	5'-d (GGAGACTACCCGGTGGATACAACT)-3'	+	171-194	PCR	
II	5'-d (GAGTTGTATCCACCGGGTAGTCTCC)-3'	-	195-171	RT + PCR	170-72 (99 nts)
	5'-d (AGGGATCCCCGGGGAAACCT)-3'	+	52-71	PCR	
III	5'-d (GGCCTCTCCTGCAGTGGTTTTTGGGGTGCCC)-3'	-	46-16	RT + PCR	15-1/246-62 (200 nts)
	5'-d (CCACTGCAGGAGAGGCCGCTTGAGGGATCCCC)-3'	+	30-61	PCR	

a The numbering of the CCCVd nucleotides is adopted from Haseloff *et al.* (1982).
b The infectious viroid RNA is (+) RNA.
c The nucleotide positions indicate the lengths of the non-primer cDNA sequence that comprise the total CCCVd sequence except for positions 51-61 but these are part of the primer sequence derived from the highly conserved central region of CCCVd.

Fig. 5.4 Ethidium bromide stained 1.2% agarose gels of PCR products from reactions using primer set III (Table 5.2) and Mu-MLV reverse transcribed cDNAs of pure $CCCVd_{246}$ as template. M denotes DNA MW marker III.

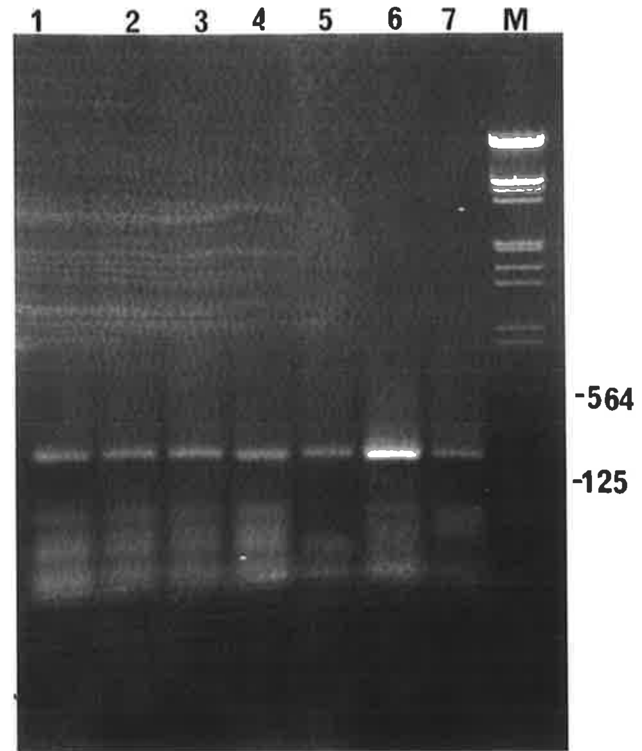
A. PCR described in Section 2.2.7b where the template cDNAs were purified by chromatography on a Sephadex column but with the following $MgCl_2$ concentrations:

1. 0.70 mM
2. 0.85 mM
3. 1.00 mM
4. 1.50 mM
5. Same as no. 4 except that reverse transcription buffer was used.
6. Same as no. 4 except that cDNA was purified by ethanol precipitation.
7. same as no. 4.

B. One-tube assay (RT-PCR) using reverse transcriptase buffer with the following purified viroid template concentrations:

1. 200 ng.
2. 100 ng.
3. 10 ng.
4. 1 ng.
5. 0.1 ng.
6. Sample as in A-4 as control.

A



B

$MgCl_2$ concentrations of 0.7 mM and 1.5 mM. Therefore, in the succeeding experiments, 1.5 mM $MgCl_2$ was used in the PCR method as described in Section 2.2.7b.

5.2.2 Development of a one-tube, one manipulation reverse transcription (RT) - PCR reaction method

Krawetz *et al.* (1989) discovered that substituting reverse transcriptase buffer for the PCR buffer markedly increases the efficiency of the PCR reaction. This was done for CCCVd and the results showed that there was minimal difference in the PCR yield between the reaction using reverse transcriptase buffer (Section 2.2.7a) and that using the recommended PCR buffer (Section 2.2.7b) (Fig. 5.4, A). However, there was an increase in the PCR yield when the first strand DNA was purified simply by ethanol precipitation before PCR rather than doing a more laborious Sephadex column purification as described in Section 2.2.6a. With these results, it was then possible to do a one-tube, RT-PCR reaction wherein reverse transcription was done first at 37°C, immediately followed by PCR in a thermal cycler after adding the two primers and *Taq* polymerase. By varying the concentration of the $CCCVd_{246}$ template, it was shown that the minimum concentration of viroid for the PCR to work under the above conditions was 100 ng (Fig. 5.4, B). This one-tube method did not work when PCR buffer was used and it was not tried on the CCCVd-related nucleic acids.

5.2.3 PCR of CCCVd-related nucleic acids

Using the standardized reverse transcription and PCR procedures done separately as described in Section 2.2.7 and the primer set I shown in Table 5.2, the circular molecules purified from the marker CCCVd extract, *M. arundinacea* and *Alpinia* sp. were tested (Fig. 5.1, A, B and C, respectively). Not one of the circular molecules from the non-palm species was amplified. Only the circular molecules 1-4 of the CCCVd extract had PCR products. The circular molecules 4 and 3 correspond to the small (246/247 nts) and large (296/297 nts) CCCVd monomers, respectively, while the circular molecules 2 and 1 are their respective dimers. The two smallest circular molecules (5 and 6) were not amplified. Molecules of about

the same size as these were also observed in the plant extracts from the non-palm species (Fig. 5.1).

When the purified isolates of the electrophoretic forms of CCCVd associated with the brooming symptoms as discussed in Section 5.1.2 were amplified, PCR products were obtained with the expected sizes as predicted from the three primer sets used (Table 5.2).

5.3 MOLECULAR CLONING OF PCR PRODUCTS

The PCR products with the electrophoretic forms of CCCVd as templates were purified by gel electrophoresis (Section 2.2.8a) to avoid the cloning of spurious bands. Polyacrylamide gel was preferred to agarose gel because it gives higher resolution. In addition, agarose is frequently contaminated with substances that are potent inhibitors of many enzymes including ligases (Maniatis *et al.*, 1982).

The Bluescript plasmid was chosen as the cloning vector because it carries lacZΔM15 and $lacI^Q$ genes on an F' episome which provide α-complementation of the β-galactosidase gene (Sambrook *et al.*, 1989). This allows blue/white color selection of recombinant colonies on plates supplemented with IPTG and X-gal.

The plasmid was prepared for ligation as described in Section 2.2.8a so that the plasmid vector and the PCR product insert had complementary single base 3' overhangs (T:A). Vector self-ligation events were prohibited by the 3' thymidine (T) overhang whereas concatamerization of the insert was prohibited by the unphosphorylated 5' end contributed by the oligonucleotide primer, as well as the 3' adenosine (A) overhang added by *Taq* polymerase during the PCR reaction (Marchuk *et al.*, 1991). Ligation of the PCR product to the vector was done as described in Section 2.2.8b.

One of the recommended *E. coli* strains for the Bluescript plasmid is JM109. Competent cells of this strain were prepared (Section 2.2.8c) and transformed with the recombinant plasmid (Section 2.2.8d). Since a small percentage of vector molecules could escape thymidine (T) addition, the recombinants were selected by employing the blue/white

color screening (Section 2.2.8e). Recombinant colonies were white. The average transformation efficiency obtained was 5.5 x 10^6 transformants/ug.

Mini-preparations (Section 2.2.9) of the recombinant plasmids were analyzed for the sizes of inserts by digestion with restriction enzymes and gel electrophoresis (Section 2.2.10). The results showed that 28 clones each had a full-length viroid insert and that 19 each had a half-length viroid insert. Fig. 5.5 shows examples of the products from restriction digests of the recombinant plasmids.

The rapid one-step-cloning procedure reported by Puchta and Sanger (1988) was tried unsuccessfully.

DISCUSSION

The polymerase chain reaction (PCR) used in this study was standardized using $CCCVd_{246}$ as the template and three sets of CCCVd-specific DNA primers. The CCCVd-like circular molecules purified by 2-D PAGE from *M. arundinacea* and *Alpinia* sp., however, were not amplified using this standardized procedure and the primer set derived from the highly conserved central region of CCCVd. It is possible that this was due to either of the following: (1) the extracts were not sufficiently pure so that a plant substance could have inhibited either the reverse transcription or the PCR reaction; (2) the circular molecules have a central domain different from CCCVd but have common sequences with CCCVd in the other domains that will explain their reaction to the CCCVd probe. No further investigations were carried out due to the limited availability of plant materials.

With the brooming-associated electrophoretic forms purified by bi-directional PAGE, amplification using the standardized PCR procedure and the three sets of primers was successful. The subsequent cloning of the PCR products using the procedures described in this chapter were also efficient. The analysis of the sizes of inserts on the recombinant plasmids by restriction enzyme digestion and PAGE revealed a total of 28 full-length clones and 19 half-length clones.

Fig. 5.5 Analysis of the sizes of the viroid inserts in the recombinant plasmids by restriction enzyme digestion and 20% non-denaturing PAGE. The gels were stained with ethidium bromide. M denotes a 123 DNA ladder.

A. Half-length viroid inserts (> 123 nts) generated by the different primer sets (Table 5.2).

Primer set I using the following templates:

1-2. Mixed isolate, nos. 7-11 (Fig. 5.3, B-1).

3. Mixed isolate, nos. 7-11 (Fig. 5.3, B-2).

4. Isolate no. 13 (Fig. 5.3, B-3).

Primer set II using the following templates:

6. Mixed isolate, nos. 7-11 (Fig. 5.3, B-1).

7. Mixed isolate, nos. 7-11 (Fig. 5.3, B-2).

12. Isolate no. 13 (Fig. 5.3, B-3).

B. Full-length viroid inserts (> 246 nts) generated by primer set III using the following templates:

1-3. Mixed isolate, nos. 7-11 (Fig. 5.3, A-2).

5-11. Mixed isolate, nos. 1-6 (Fig. 5.3, A-3).

A

B

CHAPTER 6

NUCLEOTIDE SEQUENCING OF THE SELECTED CLONES OF THE ELECTROPHORETIC FORMS OF CCCVd ASSOCIATED WITH THE SEVERE BROOMING SYMPTOMS IN COCONUT

INTRODUCTION

There are two widely used methods for DNA sequencing: the chemical method of Maxam and Gilbert (1977) and the enzymatic method of Sanger *et al.* (1977). The latter is the method of choice of most investigators as it is simpler and more convenient and it has been improved to reliably yield unambiguous results from double-stranded DNA (dsDNA) templates (Sambrook *et al.*, 1989).

In the enzymatic method, four chain-termination reactions are set up, each of which contains the template, a short oligonucleotide primer, DNA polymerase, four deoxyribonucleoside triphosphates (dNTPs) and one dideoxyribonucleoside triphosphate (ddNTP). The primer is annealed to the template and is extended by the DNA polymerase which incorporates dNTPs in a 5'→3' direction. The DNA polymerase can also incorporate a ddNTP molecule but since it lacks a 3'-OH group on the deoxyribose moiety, the ddNTP can not form a phosphodiester bond with the next dNTP in the chain. Therefore, elongation is terminated at this position. The ratio of the ddNTP to the corresponding dNTP is adjusted such that there is only partial incorporation of the ddNTP. The four sequencing reactions, therefore, yield four sets of products of different lengths, each of which has a fixed 5' end (the primer) and terminates at either A, C, G or T. Generally, the extension reaction proceeds in the presence of an α-^{32}P-dNTP. This enables the nucleotide sequence of the template to be determined after the radiolabelled products have been fractionated on a denaturing gel and subjected to autoradiography. The denaturing gel can resolve oligonucleotides which differ in length by only one nucleotide. Since all possible oligonucleotides are contained within the four sets, the complete DNA sequence can be read by loading the four sets on adjacent lanes (Sanger *et al.*, 1977; Smith, 1980).

Several different enzymes are used for dideoxy-mediated sequencing. These include the Klenow fragment of *E. coli* DNA polymerase I, reverse transcriptase, bacteriophage T7 DNA polymerases that have been modified to eliminate 3'→5' exonuclease activity (Sequenase and Sequenase Version 2.0) and the thermostable *Taq* DNA polymerase isolated from *Thermus aquaticus*. The properties of these DNA polymerases differ greatly in ways that can considerably affect the quantity and quality of the DNA sequence obtained from chain-termination reactions (Sambrook *et al.*, 1989).

Recently, automated or fluorescent DNA cycle sequencing has been developed to provide speed, accuracy and reliability. It is a variation of the Sanger method in which fluorescent labels are covalently attached to the reaction products which are then distinguished from each other by their fluorescence emission spectra collected during the polyacrylamide gel electrophoresis. This new method of sequencing can be divided into two categories: "dye primer" sequencing in which the fluorescent dyes are attached to the 5' end of the primer and "dye terminator" sequencing in which the fluorescent dyes are attached to the ddNTPs (Ciora *et al.*, 1991; Lee *et al.*, 1992). By using *Taq* DNA polymerase, the enzymatic extension reactions are performed on a DNA thermal cycler (Applied Biosystems, 1991).

The fluorescent dye primer cycle sequencing method was used in the nucleotide analysis of the electrophoretic forms of CCCVd associated with the severe brooming symptoms.

EXPERIMENTAL

6.1 EVALUATION OF THE SEQUENCING METHOD

Sequencing of a $CCCVd_{246}$ clone was first done manually using the T7 Super-Base sequencing kit manufactured by Bresatec. Apart from the hazards in handling the radiolabelled dNTPs, the enzymatic extension reactions and gel autoradiography were time-consuming and labor-intensive. The problem with "full stops", however, was the foremost drawback encountered.

With the fluorescent dye primer cycle sequencing (Section 2.2.11), "full stops" were much less frequent due to high annealing and polymerization temperatures. Furthermore, less template was required and preliminary denaturation and annealing steps were not necessary. However, both strand sequencing of a large number of clones had to be done in order to rule out PCR artefacts. The sequencing primers used based on the map of the Bluescript plasmid were T7 and M13 reverse. Although expensive, fluorescent dye primer cycle sequencing is ideal for large-scale sequencing analysis and does not have the hazards associated with radiolabelled chemicals.

6.2 SEQUENCE ANALYSIS AND SECONDARY STRUCTURE DETERMINATION

6.2.1 Forms with electrophoretic mobilities between the CCCVd variants 247 and 246

Sequence analysis (Section 2.2.12) of both strands of 22 full-length clones and seven half-length clones proved that the electrophoretic forms of CCCVd associated with the severe brooming symptoms were mutants of CCCVd. The clones generated by the mixed isolate with electrophoretic forms 247, 247a, 247b and 246 (see Fig. 5.2) also consisted of four sequence variants: the basic $CCCVd_{246}$ and three variants with 247 nts. The variants had a substitution and addition at position 197 of the central conserved domain of $CCCVd_{246}$ where C (Fig. 6.1, A) was replaced by either AU (Fig. 6.1, B), UG (Fig. 6.1, C) or UU (Fig. 6.1, D). The determination of their secondary structures (Section 2.2.12) with a minimum computed folding energy of approximately -100 kcal/mole showed that with the C→AU or C→UG exchange at position 197 in the central conserved domain, a U-loop was replaced with a base pair. The C→UU exchange, on the other hand, seemed to have a structural effect similar to that of the C→CC addition in the same 197 position of the previously described CCCVd variant 247 (Haseloff *et al.*, 1982). From the mixed isolate containing electrophoretic forms 247, 247a and 247b (Fig. 5.3, A-2), only two types of variants were cloned. One variant had 247 nts with a U→A substitution at position 216 in the P domain (Fig. 6.1, E)

A. CCCVd 246 Energy: -100.9 kcal/mole

```
                                                                                                                            *
       10            20           30           40           50            60              70          80          90         100          110          120
CU     AAAU        --   -      -CCAAAA   -   U      A     -      U     --GG      -      --GAAA  U        AA  U    A    A    UAC       U  A    -U  -    UU     G
  GGGG     CUACA  GGG CACC          ACU AC GCAGG GAG GCCGC UGAG     AUCC CCGGG            CC CAAGCG  UC GGGA GGG GCG    CUGGG CG UCG  GC GCG  GGAG A
  CCCC     GAUGU  CCC GUGG          UGA UG UGUCC UUC CGGCG ACUC     UAGG GGCCC            GG GUUCGC  AG UCCU CCC CGC    GGCCC GC AGC  CG UGC  CCUC G
-U     ----      UU   U      AAAAAAA  U  U      A     U      C     AACA      U      AUCAGA  -        -C  C    -    -    --C       -  -    UU  A    UU     A
         240          230          220        210         200         190         180         170         160             150           140         130
                                      *                    *
```

B. 247 nts Energy: -101.1 kcal/mole

```
       10            20           30           40            50             60              70          80          90         100          110          120
CU     AAAU        --   -      -CCAAAA   -   U      A     -      -      --GG      -      --GAAA  U        AA  U    A    A    UAC       U  A    -U  -    UU     G
  GGGG     CUACA  GGG CACC          ACU AC GCAGG GAG GCCGC UUGAG     AUCC CCGGG            CC CAAGCG  UC GGGA GGG GCG    CUGGG CG UCG  GC GCG  GGAG A
  CCCC     GAUGU  CCC GUGG          UGA UG UGUCC UUC CGGCG AACUC     UAGG GGCCC            GG GUUCGC  AG UCCU CCC CGC    GGCCC GC AGC  CG UGC  CCUC G
-U     ----      UU   U      AAAAAAA  U  U      A     U      U      AACA      U      AUCAGA  -        -C  C    -    -    --C       -  -    UU  A    UU     A
         240          230          220        210         200          190         180         170         160             150           140         130
                                                               **
```

C. 247 nts Energy: -100.5 kcal/mole

```
       10            20           30           40            50             60              70          80          90         100          110          120
CU     AAAU        --   -      -CCAAAA   -   U      A     -      -      --GG      -      --GAAA  U        AA  U    A    A    UAC       U  A    -U  -    UU     G
  GGGG     CUACA  GGG CACC          ACU AC GCAGG GAG GCCGCU UGAG     AUCC CCGGG            CC CAAGCG  UC GGGA GGG GCG    CUGGG CG UCG  GC GCG  GGAG A
  CCCC     GAUGU  CCC GUGG          UGA UG UGUCC UUC CGGCGG ACUC     UAGG GGCCC            GG GUUCGC  AG UCCU CCC CGC    GGCCC GC AGC  CG UGC  CCUC G
-U     ----      UU   U      AAAAAAA  U  U      A     U      U      AACA      U      AUCAGA  -        -C  C    -    -    --C       -  -    UU  A    UU     A
         240          230          220        210         200          190         180         170         160             150           140         130
                                                               **
```

D. 247 nts Energy: -100.4 kcal/mole

```
       10            20           30           40            50             60              70          80          90         100          110          120
CU     AAAU        --   -      -CCAAAA   -   U      A     -     -U      --GG      -      --GAAA  U        AA  U    A    A    UAC       U  A    -U  -    UU     G
  GGGG     CUACA  GGG CACC          ACU AC GCAGG GAG GCCGC  UGAG     AUCC CCGGG            CC CAAGCG  UC GGGA GGG GCG    CUGGG CG UCG  GC GCG  GGAG A
  CCCC     GAUGU  CCC GUGG          UGA UG UGUCC UUC CGGCG  ACUC     UAGG GGCCC            GG GUUCGC  AG UCCU CCC CGC    GGCCC GC AGC  CG UGC  CCUC G
-U     ----      UU   U      AAAAAAA  U  U      A     U     UU      AACA      U      AUCAGA  -        -C  C    -    -    --C       -  -    UU  A    UU     A
         240          230          220        210         200          190         180         170         160             150           140         130
                                                               **
```

E. 247 nts Energy: -98.9 kcal/mole

```
                                                                                              *
        10            20          30          40          50          60          70          80          90         100         110         120
CU    AAAU     --    -     -CCAAAA   -  U     A    -     U    --GG     -      --GAAA  U       AA  U    AA   A   UAC      U  A   -U  -    UU     G
  GGGG    CUACA  GGG CACC         ACU AC GCAGG GAG GCCGC UGAG     AUCC CCGGG        CC CAAGCG  UC GGGA  GGG GCG   CUGGG CG UCG  GC GCG  GGAG A
  CCCC    GAUGU  CCC GUGG         UGA UG UGUCC UUC CGGCG ACUC     UAGG GGCCC        GG GUUCGC  AG UCCU  CCC CGC   GGCCC GC AGC  CG UGC  CCUC G
-U    ----    UU   U     AAAAAAA  A  U     A   U     C     AACA     U     AUCAGA  -      -C   C     --    -    --C      -  -    UU  A   UU    A
         240        230        220         210         200         190       180          170          160          150         140        130
                                  *
```

F. 248 nts Energy: -98.4 kcal/mole

```
                                                                                              *
        10            20          30          40          50          60          70          80          90         100         110         120
CU    AAAU     --    -     -CCAAAA   -  U     A    -     -U    --GG     -      --GAAA  U       AA  U    AA   A   UAC      U  A   -U  -    UU     G
  GGGG    CUACA  GGG CACC         ACU AC GCAGG GAG GCCGC  UGAG     AUCC CCGGG        CC CAAGCG  UC GGGA  GGG GCG   CUGGG CG UCG  GC GCG  GGAG A
  CCCC    GAUGU  CCC GUGG         UGA UG UGUCC UUC CGGCG  ACUC     UAGG GGCCC        GG GUUCGC  AG UCCU  CCC CGC   GGCCC GC AGC  CG UGC  CCUC G
-U    ----    UU   U     AAAAAAA  U  U     A   U     UU     AACA     U     AUCAGA  -      -C   C     --    -    --C      -  -    UU  A   UU    A
         240        230        220         210         200         190       180          170          160          150         140        130
                                                     **
```

G. 248 nts Energy: -99.1 kcal/mole

```
                                                                                              *
        10            20          30          40          50          60          70          80          90         100         110         120
CU    AAAU     --    -     -CCAAAA   -  U     A    -     -     --GG     -      --GAAA  U       AA  U    AA   A   UAC      U  A   -U  -    UU     G
  GGGG    CUACA  GGG CACC         ACU AC GCAGG GAG GCCGC UUGAG     AUCC CCGGG        CC CAAGCG  UC GGGA  GGG GCG   CUGGG CG UCG  GC GCG  GGAG A
  CCCC    GAUGU  CCC GUGG         UGA UG UGUCC UUC CGGCG AACUC     UAGG GGCCC        GG GUUCGC  AG UCCU  CCC CGC   GGCCC GC AGC  CG JGC  CCUC G
-U    ----    UU   U     AAAAAAA  U  U     A   U     U      AACA     U     AUCAGA  -      -C   C     --    -    --C      -  -    UU  A   UU    A
         240        230        220         210         200         190       180          170          160          150         140        130
                                                     **
```

while the other variant had 248 nts with a C→UU exchange at position 197 in the central conserved domain (Fig. 6.1, F). Both variants had an A insertion at position 86 or 87 (AA→AAA) which is at the boundary of the central conserved and V domains. Two variants which were both 248 nts long, corresponding to the 247 and 247b electrophoretic forms detected in isolate no. 12 from a single palm (Fig. 5.3, A-1), again both showed the A insertion at position 86 or 87 and either the C→UU (Fig. 6.1, F) or C→AU (Fig. 6.1, G) exchange at position 197.

6.2.2 Forms with electrophoretic mobilities between and less than the CCCVd variants 297 and 296

As in a typical cadang-cadang infected palm (Haseloff *et al.*, 1982), partial sequence duplications in the V and T2 domains also occurred in the electrophoretic viroid forms as the brooming syndrome developed. However, the starting position and the length of the duplicated sequences varied and mutations of bases adjacent to the boundaries were detected. These partial sequence duplications and mutations at the boundaries were different from those described by Keese *et al.* (1988).

In Fig. 6.2, only the right-hand structures where the duplications were found in the three large electrophoretic forms cloned, are shown. Fig. 6.2(A) is the right-hand structure of the variant with 294 nts isolated singly from a brooming palm with mild stunting (isolate no. 13, Table 5.1). This variant had a U→A substitution at position 216 in the P domain of the left-hand structure (not shown). Nucleotides 98→148 (51 nts) were duplicated and inserted between positions 122 and 126 as nucleotides 123, 124 and 125 were deleted. Only two types of variants were obtained form a mixed isolate with electrophoretic forms within and above the 297 and 296 CCCVd marker region (Fig. 5.2). This was due to the decline of the transformation efficiency of the competent cells used. The left-hand structures of these variants did not have any of the mutations described above and in Section 6.2.1. The variant with 303 nts (Fig. 6.2, B) had an addition of nucleotides CGUC followed by a duplication of nucleotides 100-152 (a total of 57 nts) inserted between positions 124 and 125 in the T2 domain. The variant with 304 nts (Fig. 6.2, C) had a duplication of nucleotides 97-153 followed by a G

Fig. 6.2 Partial sequence duplications in the V and T2 domains (numbering according to that of $CCCVd_{246}$) and mutations at the boundaries of the duplicated sequences (highlighted with asterisks) in isolates from palms with the brooming symptoms. The duplicated sequences vary in positions and lengths (51, 53 and 57 nts). Note that in variant 294 (sequence A), nucleotides 123, 124 and 125 are deleted. In sequence (B), nucleotides CGUC are added before the duplicated sequences while in sequence (C), a G nucleotide is added after the duplicated sequences. Due to these mutations, structural changes are observed in the sequences.

```
A. 294 nts
                                         122
                                            98
92       98
 UAC          U  A   -U  -    UU    G    U    U  A   -U  -    UU    G
GCG   CUGGG CG UCG   GC GCG   GGA GC GGG CG UCG   GC GCG   GGAG A
CGC   GGCCC GC AGC   CG UGC   CCU CG CCC GC AGC   CG UGC   CCUC G
 --C        -  -   UU  A   UU    -    -     -  -   UU  A   UU    A
153   148                             148
                                    126
```

```
B. 303 nts
                                      124  ** **  100
92          100
 UAC         U  A   -U  GCGU           -  -       U  A   -U  -    UU    G
GCG   CUGGG CG UCG   GC     UGGAGGAG CG UCGGG CG UCG   GC GCG   GGAG A
CGC   GGCCC GC AGC   CG     GCUUCCUC GC GGCCC GC AGC   CG UGC   CCUC G
 --C        -  -   UU  --AU        A  C      -  -   UU  A   UU    A
 152                                 152
153                                 125
```

```
C. 304 nts
                                              97
          97                                124
92
 UAC          U  A   -U  -    UU    ---GA   U    U  A   -U  -    UU    G
GCG   CUGGG CG UCG   GC GCG   GGAG        GCC GGG CG UCG   GC GCG   GGAG A
CGC   GGCCC GC AGC   CG UGC   CCUC        CGG CCC GC AGC   CG UGC   CCUC G
 --C        -  -   UU  A   UU     AGCGC   -   -  -   UU  A   UU    A
153                                  *
                                125 153
```

addition (a total of 58 nts) inserted also between positions 124 and 125 in the T2 domain. The mutations at the boundaries in variants 303 and 304 had caused some changes in their secondary structures.

DISCUSSION

Fluorescent dye primer cycle sequencing proved efficient and rapid in sequencing a large number of samples. In addition, the hazards involved in handling radiolabelled chemicals are avoided as well as the tedium in the film analysis task as data collection and analysis are automated. However, it has the disadvantage of high capital cost.

Using the above sequencing method, five types of mutations in CCCVd were discovered to be associated with the severe brooming symptoms in coconut. Three of these types are variations of those previously described by Haseloff *et al.* (1982) and Keese *et al.* (1988). The five types of mutations detected are: (1) exchange of C at position 197 of the central conserved domain by either AU, UG or UU; (2) U→A substitution at position 216 in the P domain; (3) AA→AAA addition at position 86 or 87 which is at the boundary of the central conserved and V domains; (4) partial sequence duplications of the V and T2 domains at varying positions and lengths of the duplicated sequences; and (5) mutations at the boundaries of duplications which caused structural changes on the viroid molecule.

As a mutation at position 197 in the central conserved domain has been previously reported for CCCVd, that is, a C→CC addition to give the CCCVd variant 247 (Haseloff *et al.*, 1982), this site may be important for the control of pathogenicity of CCCVd. Mutations in this site may have an effect on the switching event predicted to occur during replication in which the native structure is converted into a stem loop structure (Keese *et al.*, 1988). To determine the functional implications of the new mutations in this site as well as the other types of mutations described above, infectivity trials using the mutant clones need to be carried out.

CHAPTER 7

GENERAL DISCUSSION

This thesis shows that some non-palm species in the Philippines have nucleic acids similar to the coconut cadang-cadang viroid (CCCVd) (Section 3.3.1). The nucleic acids were identified by molecular hybridization with ^{32}P-labelled cRNA and cDNA probes in dot-blots and electroblots and by the use of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). Multiple bands were found in each plant species. Those which were approximately the same size as CCCVd usually had high homology with the probe while the bands outside the CCCVd region had low homology with the probe (Sections 3.3.1 and 3.3.2a). The probable circularity of these bands was demonstrated by 2-D PAGE (Sections 3.3.2b and 5.1.1). Thus, it is concluded that the nucleic acids with CCCVd-like sequences are viroid-like, although their identity as RNA has not been confirmed by tests of nuclease sensitivity. No such sequences in non-palm species have been reported previously except by Hanold and Randles (1991) who conducted a parallel study in the south-west Pacific. However, sample collection in this thesis was more intensive and concentrated on plant species closely associated with coconut palms in the area where cadang-cadang disease occurs.

None of the non-palm species showed symptoms which could be associated with the presence of the viroid-like nucleic acids. The incidence of the viroid-like nucleic acids in these plant species was independent of the level of cadang-cadang disease in adjacent coconut plantations, and it is suggested that these plants could act as a reservoir for CCCVd-related nucleic acids in the Philippines. This would allow experiments to be done to determine whether the disease in coconut palms can be controlled by the eradication of such potential alternate sources.

Nucleotide sequencing would be expected to resolve whether the CCCVd-like nucleic acids can be classed as viroids. Since they seemed to be at a lower concentration in their hosts than CCCVd is in coconut (Section 3.2), attempts were made to amplify some of the purified isolates (Section 5.1.1) by the polymerase chain reaction (PCR) via their cDNAs. However, the conditions developed for PCR with $CCCVd_{246}$ were not successful for the

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CCCVd-like nucleic acids from the non-palm species. This could be due to either: (i) the extracts not being pure so that a plant substance could have inhibited either the reverse transcription or the PCR reaction, or (ii) the CCCVd-like nucleic acids having a central domain different from CCCVd but having common sequences with CCCVd in the other domains so as to explain their reaction to the CCCVd probe. This study was not continued because of the limited availability of plant materials. Further work should include experiments on: (i) the efficient isolation and purification of the CCCVd-like nucleic acids; (ii) the use of PCR primers derived from other parts of the CCCVd molecule; (iii) further optimization of the PCR; (iv) the use of the one-tube, reverse transcription-PCR method developed for $CCCVd_{246}$ so as to minimize losses of cDNA after reverse transcription; and (v) increasing the amounts of plant tissue to allow direct RNA sequencing.

The continuation of the study above may also lead to the possibility of finding naturally occurring variants of CCCVd in plant species other than coconut which would be useful in cross-protection studies.

The variants of CCCVd that have been reported previously are associated with disease progression but not with unusual types of symptom (Sections 1.2.4 and 1.2.5). All of these variants contain the basic 246 nucleotides. In Chapter 4, the appearance of a new unusually severe brooming symptom in palms infected by artificial inoculation with preparations of CCCVd is discussed. "Brooming" is a severe form of cadang-cadang disease characterized by a loss of leaf lamina, severe stunting and occasional premature death. Approximately 12% of 1,787 CCCVd-inoculated palms screened showed brooming. Observed 3-7 years after inoculation, it was essentially confined to palms with either CCCVd variant 246 or 247.

PAGE and molecular hybridization analyses of 136 palms with "brooming" showed that all of them have electrophoretic forms distinct from the previously reported CCCVd variants (Section 4.2). Whereas four bands representing viroids with 246, 247, 296 and 297 nts are observed in extracts from palms with common cadang-cadang disease, different palms with brooming symptoms showed a total of 11 additional or replacement bands. On 20% PAGE, three of these bands had electrophoretic mobilities between CCCVd variant 247 and 246; two, between 246 and 297; three, between 297 and 296, and three, less than 296. To

determine whether these electrophoretic forms are mutants of CCCVd, some isolates representing most of the above additional or replacement bands were cloned and sequenced. Cloning had to be done since most of the isolates contained more than one electrophoretic form making it difficult to relate a particular form to a particular symptom as well as to analyze data generated by direct sequencing.

As described in Chapter 5, isolates with a mixture of brooming-associated electrophoretic forms were first purified by bi-directional PAGE (Section 5.1.2) and then amplified by PCR via their cDNAs (Section 5.2.3). Attempts to separate each form from the others in the bi-directional PAGE failed. The design of primers in the synthesis of cDNAs and their subsequent amplification of PCR is an important factor to consider. In Section 5.2.1a, three sets of primers were used to polymerase almost the whole CCCVd sequence so as not to miss any sites of mutation: (i) primer set I to produce the left half-length of the total CCCVd sequence, (ii) primer set II to produce the right half-length, and (iii) primer set III to generate full-length fragments. With primer sets I and II, amplification was successful only when the synthesis of the first strand cDNA was done using the AMV reverse transcription method. On the other hand, full-length CCCVd was amplified using primer set III and the M-MuLV reverse transcriptase method. The priming sites for primer sets I and II are at the highly conserved central region of CCCVd where there is more secondary structure and more G-C base pairs than in the priming site for primer set III which is at the upper left portion of the molecule. Thus, it is concluded that AMV reverse transcriptase method must have provided the right conditions for the melting of the viroid and annealing of the primers.

PCR products with the expected sizes were cloned by inserting them into the Bluescript plasmid vector with the use of single base A:T overlaps (Section 5.3), a method that has the efficiency of a sticky-end cloning. A total of 28 full-length clones and 19 half-length clones were obtained. Only 27 were selected for sequence analysis.

Sequencing of the selected clones was done by fluorescent dye primer cycle sequencing (Sections 6.1 and 6.2). This method proved efficient and rapid in sequencing a large number of samples. Moreover, the hazards involved in handling radiolabelled chemicals were avoided as well as the tedium in the final analysis task as data collection and analysis were

automated. However, sequencing of both strands of a large number of clones had to be done in order to rule out PCR artefacts.

The sequencing data revealed the following types of mutation observed in the clones in addition to the basic $CCCVd_{246}$: (i) substitution of C at position 197 of the central conserved domain with either AU, UG or UU; (ii) substitution of U with A at position 216 in the P domain; (iii) addition of A at position 86 or 87 so that AA became AAA at the boundary of the central conserved region and the V domain; (iv) mutants of varying length resulting from partial sequence duplications of the V and T2 domains commencing from varying positions in the right-hand end of the viroid molecule; and (v) mutations at the boundaries of duplications. Mutations (i), (iv) and (v) are variations of those previously described by Haseloff *et al.* (1982) and Keese *et al.* (1988).

Determination of optimal secondary structures showed that with the C→AU or C→UG substitution at position 197 in the central conserved domain, a U-loop was replaced with a base pair. Also, mutations at the boundaries of partial sequence duplications could cause structural changes in the V and T2 domains. The other mutations appeared not to affect secondary structure. Previous observation, however, has been reported that although a C→CC addition at position 197 of the central conserved region appears not to affect secondary structure, it causes the CCCVd molecule to migrate with markedly different electrophoretic mobility from the basic $CCCVd_{246}$ in which there is no added cytosine residue (Haseloff *et al.*, 1982; Imperial and Rodriguez, 1983). Thus, the new variant discovered in this thesis with the C→UU substitution at position 197, could have the same structural effect as that of the above CCCVd variant with 247 nts.

As position 197 of the central conserved region seemed to be vulnerable to mutations with the resulting variants having different mobilities in PAGE, this site may be important for the control of pathogenicity of CCCVd. Mutations in this site may have an effect on the switching event predicted to occur during replication in which the native structure is converted into a stem loop structure (Keese *et al.*, 1988).

In two of the sequence variants with partial sequence duplications of the V and T2 domains, no mutation was observed in the left hand-portion of the native molecules. This was despite the presence of small monomers with mutations in the said portion. Haseloff *et al.*

(1982) and Imperial and Rodriguez (1983) have both reported that occasionally, when additions of the duplicated molecular segment occur, only $CCCVd_{296}$ (without cytosine) appears despite the presence of $CCCVd_{247}$ (with cytosine). However, the third large mutant which was isolated singly from a palm with "brooming" but with mild stunting, had a U→A substitution at position 216 in the P domain of the left-hand structure aside from partial sequence duplication of the V and T2 domains at the right-hand portion of the molecule.

It will only be possible to determine whether there is a direct causal relationship between the mutations and "brooming" by inoculating the mutant clones to coconut palms. This will determine what particular type or combination of mutations causes the severe symptoms. This may identify sites in the CCCVd molecule where *in vitro* mutagenesis can be induced to modify the pathogenicity of the viroid.

Sequencing of the other electrophoretic forms associated with "brooming" as described in Section 4.2 could reveal more novel types of mutation in CCCVd.

The use of molecular biology techniques in this study has demonstrated that CCCVd-related sequences are widespread in a range of non-palm plant species. The possibility arises that these plants could act as sources of infection for coconuts in the field. Furthermore, elucidation of the molecular basis of the unusually severe "brooming" symptoms observed in coconuts has been made possible. This will be useful in re-evaluating the distribution of functions between the conserved and variable features of the CCCVd molecule in comparison with previously described models for other viroids (Keese *et al.*, 1987). These molecular studies will assist in developing appropriate control measures for CCCVd.

APPENDIX A

BIOCHEMICAL	SOURCE
dATP	Boehringer, Mannheim, FRG
dGTP	Boehringer, Mannheim, FRG
dCTP	Boehringer, Mannheim, FRG
dTTP	Boehringer, Mannheim, FRG
EcoRV	Boehringer, Mannheim, FRG
T_4DNA ligase	Boehringer, Mannheim, FRG
RNAse A (DNAse-free)	Boehringer, Mannheim, FRG
DNA molecular weight marker III	Boehringer, Mannheim, FRG
EcoRI	Boehringer, Mannheim, FRG
Hind III	Boehringer, Mannheim, FRG
BamHI	Boehringer, Mannheim, FRG
RNAsin	Bresatec, Australia
a-^{32}S-dATP	Bresatec, Australia
T7 Super-Base sequencing kit	Bresatec, Australia
a-^{32}P-dCTP	Amersham International, England
M-MuLV reverse transcriptase	Bethesda Research Laboratories, USA
AMV reverse transcriptase	Pharmacia LKB Biotechnology, Sweden
Taq DNA polymerase PCR kit	Promega
123 DNA ladder	GIBCO, BRL
Taq dye primer cycle sequencing kit	Applied Biosystems, Inc.

CHEMICAL	SOURCE
Cetythrimethylammonium bromide (CTAB)	Sigma, USA
Polyethylene glycol (PEG) 6000	Sigma, USA
Sodium dodecyl sulphate (SDS)	Sigma, USA
Mineral oil for PCR	Sigma, USA
Dimethyl sulfoxide (DMSO)	Sigma, USA
Ethidium bromide	Sigma, USA
Agarose Type II: medium EEO	Sigma, USA
Acrylamide	Bio-Rad Laboratories, USA
N, N'-Methylene-bis-acrylamide	Bio-Rad Laboratories, USA
N, N, N'-N'-Tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories, USA
G 50 (fine) - Sephadex	Pharmacia, Sweden
Ficoll 400	Pharmacia, Sweden
Polyvinylpyrrolidone (PVP)	Sigma, USA
Dextran sulphate	Pharmacia, Sweden
Isopropyl-b-D-thiogalactopyranoside (IPTG)	Boehringer, Mannheim
5-Bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal)	Boehringer, Mannheim
Silver nitrate ($AgNO_3$)	BDH Chemicals, Australia
Sodium borohydride ($NaBH_4$)	Sigma, USA
Formaldehyde	Ajax Chemicals Ltd., Australia
Formamide	Sigma, USA
Lithium chloride (LiCl)	Sigma, USA
2-(N-Morpholino) ethanesulfonic acid (MES)	Sigma, USA
Purified agar	Oxoid Ltd., England
Bacto-tryptone	Oxoid Ltd., England
Bacto-yeast extract	Oxoid Ltd., England
Ilford rapid fixer	Ilford Pty. Ltd., Australia
Kodak developer	Kodak Pty. Ltd.
Phenol	BDH Chemicals, Australia

APPENDIX B

PHENOL REAGENTS

For RNA extraction:	90% phenol in deionized water with 0.1% 8-hydroxyquinoline
For DNA extraction:	Liquified phenol equilibrated in equal volume of TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA)

POLYACRYLAMIDE GELS

7% non-denaturing gel		20 ml solution
40% acrylamide (3% bis)	=	3.5 ml
10 X TBE	=	2.0
deionized water	=	13.892
TEMED	=	0.008
10% ammonium persulphate (AP)	=	0.6

7% denaturing gel (8 M urea)		20 ml solution
40% acrylamide (3% bis)	=	3.5 ml
10 X TBE	=	2.0
deionized water	=	7.0
TEMED	=	0.008
10% AP	=	0.6
Urea	=	9.6 g

20% non-denaturing gel 20 ml solution

40% acrylamide (3% bis)	=	10.0 ml
10 X TBE	=	2.0
deionized water	=	7.192
TEMED	=	0.008
10% AP	=	0.8

AGAROSE GEL

1.2% agarose (Type II: medium EEO) in TAE buffer (40 mM Tris-acetate; 1 mM EDTA) with 1 ppm ethidium bromide.

BACTERIAL MEDIA

LB medium

Per liter:

To 950 ml of deionized water, add:

bacto-tryptone	10 g
bacto-yeast extract	5 g
NaCl	10 g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume of the solution to 1 liter with deionized water. Sterilize by autoclaving for 20 min at 15 lb/sq. in. on liquid cycle.

SOB medium

Per liter:

To 950 ml of deionized water, add:

bacto-tryptone	20 g
bacto-yeast extract	5 g
NaCl	0.5 g

Shake until the solutes have dissolved. Add 10 ml of a 250 mM solution of KCl (this solution is made by dissolving 1.86 g of KCl in 100 ml of deionized water). Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume of the solution to 1 liter with deionized water. Sterilize by autoclaving for 20 min at 15 lb/sq. in. on liquid cycle.

Just before use, add 5 ml of a sterile solution of 2 M $MgCl_2$. This solution is made by dissolving 19 g of $MgCl_2$ in 90 ml deionized water. Adjust the volume of the solution to 100 ml with deionized water and sterilize by autoclaving as above.

SOC medium

SOC medium is identical to SOB medium, except that it contains 0.35% glucose. After the SOB medium has been autoclaved, allow it to cool to 60°C or less and then add 7 ml of a sterile 50% solution of glucose (this solution is made by dissolving 50 g of glucose in 90 ml of deionized water). After the sugar has dissolved, adjust the volume of the solution to 100 ml with deionized water and sterilize by filtration through a 0.22-micron filter).

Storage of bacterial culture containing glycerol

To 0.85 ml of bacterial culture, add 0.15 ml of sterile gylcerol. Vortex the culture to ensure that the glycerol is evenly dispersed and transfer to a labelled storage tube equipped with a screw cap. Freeze the culture in liquid nitrogen and then transfer the tube to -70°C for long-term storage.

APPENDIX C

The provinces (shaded areas) in the central Philippines with cadang-cadang infected coconut plantations. The site codes listed in Table 3.1 are: (A) Pili, Bacacay, Albay; (B) Albay Research Center; (C) Pili, Camarines Sur; (D) Langa, Camarines Sur; (E) Sangay, Camarines Sur; (F) Atulayan, Camarines Sur; (G) Infanta, Quezon; (H) Calabanga, Camarines Sur; (I) Tublejon, Sorsogon; (J) Cogon, Sorsogon; and (K) Lipa, Batangas.

REFERENCES

Anonymous (1986) Annual Report 1986 of the Philippine Coconut Authority, Agricultural Research and Development Branch.

Applied Biosystems (1991) *Dye Primer Cycle Sequencing* Part No. 901482/Rev. B.

Applied Biosystems (1991) *User Bulletin for DNA Sequencer.* Model 373A No. 18.

Ashulin, L, Lachman, O, Hadas, R and Bar-Joseph, M (1991) Nucleotide sequence of a new viroid species, citrus bent leaf viroid (CBLVd) isolated from grapefruit in Israel. *Nucl. Acids Res.* **19(17):** 4767.

Ausubel, FM, Brent, R, Kingston, RE, Moore, DD, Seidman, JG, Smith, JA and Struhl, K (1991) The polymerase chain reaction. Pages 15.0.3-15.4.5 in: *Current Protocols in Molecular Biology.* John Wiley and Sons, USA.

Barker, JM, McInnes, JL, Murphy, PJ and Symons, RH (1985) Dot-blot procedure with [^{32}P] DNA probes for the sensitive detection of avocado sunblotch and other viroids in plants. *J. Virol. Methods* **10:** 87-98.

Boccardo, G, Beaver, RG, Randles, JW and Imperial, JS (1981) Tinangaja and bristle top, coconut diseases of uncertain etiology in Guam and their relationship to cadang-cadang disease of coconut in the Philippines. *Phytopathology* **71:** 1104.

Branch, AD and Robertson, HD (1984) A replication cycle for viroids and other small infectious RNAs. *Science* **223:** 450-455.

Branch, AD, Benenfeld, BJ, Franck, ER, Shaw, JF, Varban, ML, Willis, KK, Rosen, DL and Robertson, HD (1988) Interference between coinoculated viroids. *Virology* **163:** 538-546.

Candresse, T, Diener, TO and Owens, RA (1990) The role of the viroid central conserved region in cDNA infectivity. *Virology* **175:** 232-237.

Candresse, T, Smith, D and Diener, TO (1987) Nucleotide sequence of a full-length infectious clone of the Indonesian strain of tomato apical stunt viroid (TASV). *Nucl. Acids Res.* **15(24):** 10597.

Chien, A, Edgar, DB and Trela, JM (1976) Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus. J. Bacteriology* **127(3):** 1550-1557.

Ciora, T, Denéfle, P and Mayaux, JF (1991) Rapid one-step automated sequencing reactions for 16 DNA samples using *Taq* polymerase and fluorescent primers. *Nucl. Acids Res.* **19(1):** 188.

Clark, JM (1988) Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucl. Acids Res.* **16(20):** 9677-9686.

Cress, DE, Kiefer, MC and Owens, RA (1983) Construction of infectious potato spindle tuber viroid cDNA clones. *Nucl. Acids Res.* **11(19):** 6821-6835.

Dahlgren, RMT, Clifford, HT and Yeo, PF (1985) *The Families of the Monocotyledons: Structure, Evolution and Taxonomy.* 520 pp. Springer-Verlag, Berlin.

Diener, TO (1971) Potato spindle tuber "virus". IV. A replicating, low molecular weight RNA. *Virology* **45:** 411-428.

Diener, TO (1979) *Viroids and Viroid Diseases.* John Wiley and Sons, New York.

Diener, TO (1987) Biological properties. Pages 9-29 in: *The Viroids.* TO Diener, ed. Plenum Press, New York.

Diener, TO (1991) Subviral pathogens of plants: Viroids and viroid-like satellite RNAs. *FASEB J.* **5:** 2808-2813.

Galindo, J, Lopez, C and Aguilar, T (1989) Discovery of the transmitting agent of tomato planta macho viroid. *Revista Mexicana de Fitopatologia* **7:** 61-65.

Gross, HJ, Domdey, H, Lossow, C, Jank, P, Raba, M, Alberty, H and Sanger, HL (1978) Nucleotide sequence and secondary structure of potato spindle tuber viroid. *Nature* (London) **273:** 203.

Gross, HJ, Krupp, G, Domdey, H, Raba, M, Jank, P, Lossow, C, Alberty, H, Ramm, K and Sanger, HL (1982) Nucleotide sequence and secondary structure of citrus exocortis and chrysanthemum stunt viroid. *Eur. J. Biochem.* **121:** 249.

Haas, B, Klanner, A, Ramm, K and Sanger, HL (1988) The 7S RNA from tomato leaf tissue resembles a signal recognition particle RNA and exhibits a remarkable sequence complementarity to viroids. *EMBO J.* **7(13):** 4063-4074.

Hammond, R, Smith, DR and Diener, TO (1989) Nucleotide sequence and proposed secondary structure of columnea latent viroid: A natural mosaic of viroid sequences. *Nucl. Acids Res.* **17:** 10083-10094.

Hammond, RW and Owens, RA (1987) Mutational analysis of PSTVd reveals complex relationships between structure and infectivity. *Proc. Natl. Acad. Sci. USA* **84:** 3967-3971.

Hammond, RW, Diener, TO and Owens, RA (1989) Infectivity of chimeric viroid transcripts reveals the presence of alternative processing sites in PSTVd. *Virology* **170:** 486-495.

Hanahan, D (1983) Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166:** 557-580.

Hanold, D and Randles, JW (1991) Coconut cadang-cadang disease and its viroid agent. *Plant Disease* **75(4):** 330-335.

Hanold, D and Randles, JW (1991) Detection of the cadang-cadang viroid-like sequences in oil and coconut palm and other monocotyledons in the south-west Pacific. *Ann. appl. Biol.* **118:** 139-151.

Hanold, D, Rodriguez, MJB and Randles, JW (1989) Detection of sequences related to coconut cadang-cadang viroid in new species and in new locations. In Proceedings, 7th Australasian Plant Pathology Society Conference, Brisbane, p. 73.

Haseloff, J, Mohamed, NA and Symons, RH (1982) Viroid RNAs of cadang-cadang disease of coconuts. *Nature* **299:** 316-321.

Hernandez, C, Elena, SF, Moya, A and Flores, R (1992) Pear blister canker viroid is a member of the apple scar skin subgroup (apscaviroids) and also has sequence homology with viroids from other subgroups. *J. gen. Virol.* **73:** 2503-2507.

Herold, T, Haas, B, Singh, RP, Boucher, A and Sanger, HL (1992) Sequence analysis of five new field isolates demonstrates that the chain length of potato spindle tuber viroid (PSTVd) is not strictly conserved but as variable as in other viroids. *Plant Mol. Biol.* **19:** 329-333.

Holton, TA and Graham, MW (1991) A simple and efficient method for direct cloning of PCR products using ddT-tailed vectors. *Nucl. Acids Res.* **19:** 1156.

Imperial, JS and Rodriguez, MJB (1983) Variation in the coconut cadang-cadang viroid: Evidence for single base addition with disease progress. *Philipp. J. Crop Sci.* **8(2):** 87-91.

Imperial, JS, Bautista, RM and Randles, JW (1985) Transmission of the coconut cadang-cadang viroid to six species of palm by inoculation with nucleic acid extracts. *Plant Pathology* **34:** 391-401.

Imperial, JS, Rodriguez, MJB and Randles, JW (1981) Variation in the viroid-like RNA associated with cadang-cadang disease: Evidence for an increase in molecular weight with disease progress. *J. gen. Virol.* **56:** 77-85.

Ishikawa, M, Meshi, T, Okada, Y, Sano, T and Shikata, E (1985) *In vitro* mutagenesis of infectious viroid cDNA clone. *J. Biochem.* **98(6):** 1615-1620.

Keese, P and Symons, RH (1985) Domains in viroids: Evidence of intermolecular RNA managements and their contribution to viroid evolution. *Proc. Natl. Acad. Sci. USA* **82:** 4582-4586.

Keese, P and Symons, RH (1987) The structure of viroids and virusoids. Pages 1-47 in: *Viroids and Viroid-like Pathogens*. JS Semancik, ed. CRC Press, Boca Raton, FL.

Keese, P, Osorio-Keese, ME and Symons, RH (1988) Coconut tinangaja viroid: Sequence homology with coconut cadang-cadang viroid and other potato spindle tuber viroid related RNAs. *Virology* **162:** 508-510.

Keese, P, Visvader, JE and Symons, RH (1988) Sequence variability in plant viroid RNAs. Pages 71-98 in: *RNA Genetics, Variability of RNA Genomes*, Vol. III. E. Domingo, JJ Holland and P Ahlquist, eds. CRC Press, Boca Raton, FL.

Khoury, J, Singh, RP, Boucher, A and Coombs, DH (1988) Concentration and distribution of mild and severe strains of potato spindle tuber viroid in cross-protected tomato plants. *Phytopathology* **78(10):** 1331-1336.

Koltunow, AM and Rezaian, MA (1989) A scheme for viroid classification. *Intervirology* **30:** 194-201.

Krawetz, SA, Pon, RT and Dixon, GH (1989) Increased efficiency of the *Taq* polymerase catalyzed polymerase chain reaction. *Nucl. Acids Res.* **17(2):** 819.

Kwok, S and Higuchi, R (1989) Avoiding false positives with PCR. *Nature* **339:** 237-238.

Laulhere, J and Rozier, C (1976) One-step extraction of plant nucleic acids. *Plant Science Letters* **6:** 237-242.

Lee, LG, Commell, CR, Woo, SL, Cheng, RD, McArdle, BF, Fuller, CW, Halloran, ND and Wilson, RK (1992) DNA sequencing with dye-labelled terminations and T7 DNA polymerase: Effect of dyes and dNTPs on incorporation of dye-terminators and probability analysis of termination fragments. *Nucl. Acids Res.* **20(10):** 2471-2483.

Lowe, T, Sharefkin, J, Yang, SQ and Dieffenbach, CW (1990) A computer program for selection of oligonucleotide primers for polymerase chain reaction. *Nucl. Acids Res.* **18(7):** 1757-1761.

Maniatis, T, Fritsch, EF and Sambrook, J (1982) *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Laboratory Press, USA.

Marchuk, D, Drumm, M, Sauline, A and Collins, FS (1991) Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucl. Acids Res.* **19(5):** 1154.

Maxam, AM and Gilbert, W (1977) A new method for sequencing DNA. *Proc. Natl. Acad. Sci.* **74:** 560.

Maxam, AM and Gilbert, W (1980) In *Methods in Enzymology* **65(1):** 499-560. L Grossman and K Moldave, eds. Academic Press, New York.

McInnes, JL and Symons, RH (1989) Nucleic acid probes in the diagnosis of plant viruses and viroids. Pages 113-138 in: *Nucleic Acid Probes.* RH Symons, ed. CRC Press, Inc., Boca Raton, Florida.

Mead, DA, Pey, NK, Herrnstadt, C, Marcil, RA and Smith, LM (1991) A universal method for the direct cloning of PCR amplified nucleic acid. *Bio/Technology* **9:** 657-663.

Meshi, T, Ishikawa, M, Ohno, T, Okada, Y, Sano, T, Ueda, I and Shikata, E (1984) Double-stranded cDNAs of hop stunt viroid are infectious. *J. Biochem.* **95(5):** 1521-1524.

Meshi, T, Ishikawa, M, Watanabe, Y, Yamaya, J, Okada, Y, Sano, T and Shikata, E (1985) The sequence necessary for the infectivity of hop stunt viroid cDNA clones. *Mol. Gen. Genet.* **200:** 199-206.

Mohamed, NA, Bautista, R, Buenaflor, G and Imperial, JS (1985) Purification and infectivity of the coconut cadang-cadang viroid. *Phytopathology* **75(1):** 79-83.

Mohamed, NA, Bautista, R, Buenaflor, G and Imperial, JS (1985) Purification and infectivity of the coconut cadang-cadang viroid. *Phytopathology* **75(1):** 79-83.

Mohamed, NA, Haseloff, J, Imperial, JS and Symons, RH (1982) Characterization of the different electrophoretic forms of the cadang-cadang viroid. *J. gen. Virol.* **63:** 181-188.

Moody, K, Munroe, CE, Lubigan, RT and Paller, EC (1984) Major Weeds of the Philippines. *Weed Science Society of the Philippines*, UPLB.

Niblett, CL, Dickson, E, Fernow, KH, Horst, RK and Zaitlin, M (1978) Cross protection among four viroids. *Virology* **91:** 198-203.

Ocfemia, GO (1937) The probably nature of cadang-cadang disease of coconuts. *Philippine Agri.* **26:** 338-340.

Ohno, T, Takamatsu, N, Meshi, T and Okada, Y (1983) Hop stunt viroid: Molecular cloning and nucleotide sequence of the complete cDNA copy. *Nucl. Acids Res.* **11:** 6185.

Owens, RA and Hammond, RW (1990) Mutational analysis of viroid pathogenicity and movement. Pages 165-176 in: *Viral genes and plant pathogenesis*. TP Pirone and TG Shaw, eds. Springer-Verlag, New York.

Owens, RA and Hammond, RW (1990) Mutational analysis of viroid. *Seminars in Virology* **1:** 101-106.

Owens, RA, Candresse, T and Diener, TO (1990) Construction of novel viroid chimeras containing portions of tomato apical stunt and citrus exocortis viroids. *Virology* **175:** 238-246.

Owens, RA, Hammond, RW, Gardner, RC, Kiefer, MC, Thompson, SM and Cress, DE (1986) Site-specific mutagenesis of potato spindle tuber viroid cDNA: Alterations within premelting region 2 that abolish infectivity. *Plant Mol. Biol.* **6:** 179-192.

Owens, RA, Khurana, SMP, Smith, DR, Singh, MN and Garg, ID (1992) A new mild strain of potato spindle tuber viroid isolated from wild *Solanum* spp. in India. *Plant Disease* **76(5):** 527-529.

Owens, RA, Thompson, SM and Steger, G (1991) Effects of random mutagenesis upon potato spindle tuber viroid replication and symptom expression. *Virology* **185:** 18-31.

Pacumbaba, EP and Carpio, CB (1987) Report on cadang-cadang surveys in western and eastern Samar. Philippine Coconut Authority, Albay Research Center.

Peacock, AC and Dingman, CW (1968) Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide gels. *Biochemistry* **7:** 668-674.

Price, WC (1971) Cadang-cadang of coconut - A review. *Plant Science* **3:** 1-13.

Puchta, H and Sanger, HL (1988) An improved procedure for the rapid one-step-cloning of full-length viroid cDNA. *Arch. Virol.* **101:** 137-140.

Puchta, H and Sanger, HL (1989) Sequence analysis of minute amounts of viroid RNA using the polymerase chain reaction (PCR). *Arch. Virol.* **106:** 335-340.

Puchta, H, Herold, T, Verhoeven, K, Roenhorst, A, Ramm, K, Schmidt-Puchta, W and Sanger, HL (1990) A new strain of potato spindle tuber viroid (PSTVd-N) exhibits major sequence differences as compared to all other PSTVd strains sequenced so far. *Plant Mol. Biol.* **15:** 509-511.

Puchta, H, Ramm, K and Sanger, HL (1988) The molecular structure of hop latent viroid (HLVd), a new viroid occurring worldwide in hops. *Nucl. Acids Res.* **16(10):** 4197-4216.

Randles, JS, Boccardo, G and Imperial, JS (1980) Detection of the cadang-cadang RNA in African oil palm and buri palm. *Phytopathology* **70:** 185-189.

Randles, JW (1975) Association of two ribonucleic acid species with cadang-cadang disease of coconut palm. *Phytopathology* **65:** 163-167.

Randles, JW (1987) Coconut cadang-cadang. Pages 265-277 in: *The Viroids*. TO Diener, ed. Plenum Press, New York.

Randles, JW and Hatta, T (1979) Circularity of the ribonucleic acids associated with cadang-cadang disease. *Virology* **96:** 47-53.

Randles, JW and Palukaitis, P (1979) *In vitro* synthesis and characterization of DNA complementary to cadang-cadang-associated RNA. *J. gen. Virol.* **43:** 649-662.

Randles, JW, Boccardo, G, Retuerma, ML and Rillo, ES (1977) Transmission of the RNA species associated with cadang-cadang of coconut palm, and insensitivity of the disease to antibiotics. *Phytopathology* **67:** 1211-1216.

Randles, JW, Hanold, D, Pacumbaba, EP and Rodriguez, MJB (1992) Cadang-cadang disease of coconut palm. Pages 277-295 in: *Plant Diseases of International Importance*. AN Mukhopadhyay, J Kumar, HS Chaube and US Singh, eds. Prentice Hall Inc., New Jersey.

Randles, JW, Rillo, EP and Diener, TO (1976) The viroid-like structure and cellular location of anomalous RNA associated with the cadang-cadang disease. *Virology* **74:** 128-139.

Randles, JW, Rodriguez, MJB and Imperial, JS (1988) Cadang-cadang disease of coconut palm. *Microbiological Sciences* **5(1):** 18-22.

Randles, JW, Steger, G and Riesner, D (1982) Structural transitions in viroid-like RNAs associated with cadang-cadang disease, velvet tobacco mottle virus, and *Solanum nodiflorum* mottle virus. *Nucl. Acids Res.* **10(18):** 5569-5585.

Rezaian, MA (1990) Australian grapevine viroid - evidence for extensive recombination between viroids. *Nucl. Acids Res.* **18:** 1813-1818.

Riesner, D (1987) Subcellular location and *in situ* association with cellular components. Pages 99-116 in: *The Viroids*. TO Diener, ed. Plenum Press, New York.

Riesner, D, Steger, G, Schumacher, J, Gross, HJ, Randles, JW and Sänger, HL (1983) Structure and function of viroids. *Biophys. Struct. Mech.* **9:** 145.

Rigden, JE and Rezaian, MA (1992) *In vitro* synthesis of an infectious viroid: Analysis of the infectivity of monomeric linear CEVd. *Virology* **186:** 201-206.

Rillo, EP and Rillo, AR (1981) Abstracts on the cadang-cadang disease of coconut (1937-1980). Philippine Coconut Authority.

Rivera-Bustamante, RF, Gin, R and Semancik, JS (1986) Enhanced resolution of circular and linear molecular forms of viroid and viroid-like RNA by electrophoresis in a discontinuous-pH system. *Analytical Biochemistry* **156:** 91-95.

Ruano, G, Brash, DE and Kidd, KK (1991) PCR: The first few cycles. *Perkin Elmer Cetus' Amplifications, A Forum for PCR Users.* Issue **7:** 1-4.

Saiki, RK, Gelfand, DH, Stoffel, S, Scharf, SJ, Higuchi, R, Horn, GT, Mullis, KB and Erlich, HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239:** 487-491.

Sambrook, J, Fritsch, EF and Maniatis, T (1989) DNA sequencing. Pages 13.3-13.95 in: *Molecular Cloning, A Laboratory Manual,* Cold Spring Harbor Laboratory Press, USA.

Sambrook, J, Fritsch, EF and Maniatis, T (1989) *In vitro* amplification of DNA by the polymerase chain reaction. Pages 14.2-14.35 in: *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Laboratory Press, USA.

Sambrook, J, Fritsch, EF and Maniatis, T (1989) *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

Sammons, DW, Adams, LD and Nishazawa, EE (1981) Ultrasensitive silver-based color staining of polypeptides in polyacrylamide gels. *Electrophoresis* **2:** 135-141.

Sanger, F, Nicklen, FS and Coulson, AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74:** 5463.

Sano, T, Candresse, T, Hammond, RW, Diener, TO and Owens, RA (1992) Identification of multiple structural domains regulating viroid pathogenicity. *Proc. Natl. Acad. Sci. USA* **89:** 10104-10108.

Sano, T, Uyeda, I, Shikata, E, Ohno, T and Okada, Y (1984) Nucleotide sequence of cucumber pale fruit viroid: Homology to hop stunt viroid. *Nucl. Acids Res.* **12:** 2437.

Sarkar, G and Sommer, SS (1990) Shedding light on PCR contamination. *Nature* **343:** 27.

Schnölzer, M, Haas, B, Ramm, K, Hofmann, H and Sanger, HL (1985) Correlation between structure and pathogenicity of potato spindle tuber viroid (PSTV). *EMBO J.* **4:** 2181-2199.

Schumacher, J, Randles, JW and Riesner, D (1983) A two-dimensional electrophoretic technique for the detection of circular viroids and virusoids. *Analytical Biochemistry* **135:** 288-295.

Singh, RP, Boucher, A and Somerville, TH (1992) Detection of potato spindle tuber viroid in the pollen and various parts of potato plant pollinated with viroid-infected pollen. *Plant Disease* **76(9):** 951-953.

Smith, AJH (1980) DNA sequence analysis by primed synthesis. *Methods Enzymol.* **65:** 560.

Symons, RH (1981) Avocado sunblotch viroid: Primary sequence and proposed secondary structure. *Nucl. Acids Res.* **9:** 6527.

Symons, RH (1991) The intriguing viroids and virusoids: What is their information content and how did they evolve? *Molecular Plant-Microbe Interactions* **4(2):** 111-121.

Symons, RH, Haseloff, J, Visvader, JE, Keese, P, Murphy, PJ, Gill, DS, Gordon, KHJ and Bruening, G (1985) On the mechanism of replication of viroids, virusoids and satellite RNAs. Pages 235-263 in: *Subviral Pathogens of Plants and Animals: Viroids and Prions.* K Maramorosch and JJ McKelvey, eds. Academic Press, New York.

Tabler, M and Sanger, HL (1984) Cloned single- and double-stranded DNA copies of potato spindle tuber viroid (PSTV) RNA and co-inoculated subgenomic DNA fragments are infectious. *EMBO J.* **3:** 3055-3062.

Tabler, M and Sanger, HL (1985) Infectivity studies on different potato spindle tuber viroid (PSTV) RNAs synthesized *in vitro* with the SPC transcription system. *EMBO J.* **4(9):** 2191-2199.

Tabler, M, Günther, I, Kern, R and Sanger, HL (1989) A microscale procedure for isolating and sequencing the viroid RNA present in one gram of infected leaf tissue. *J. Virol. Methods* **23:** 111-126.

Thomas, P (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad.Sci., USA* **77:** 5201-5205.

Titus, DE, ed. (1991) *Promega Protocols and Applications Guide.*

Tsagris, M, Tabler, M, Mühlbach, HP and Sanger, HL (1987) Linear oligomeric potato spindle tuber viroid (PSTVd) RNAs are accurately processed *in vitro* to the monomeric circular viroid proper when incubated with a nuclear extract from healthy potato cells. *EMBO J.* **6:** 2173-2183.

Visvader, JE and Symons, RH (1985) Eleven new sequence variants of citrus exocortis viroid and the correlation of sequence with pathogenicity. *Nucl. Acids Res.* **13:** 2907-2920.

Visvader, JE and Symons, RH (1986) Replication of *in vitro* constructed viroid mutants: Location of the pathogenicity-modulating domain of citrus exocortis viroid. *EMBO J.* **5(9):** 2051-2055.

Visvader, JE, Forster, AC and Symons, RH (1985) Infectivity and *in vitro* mutagenesis of monomeric cDNA clones of citrus exocortis viroid indicates the site of processing of viroid precursors. *Nucl. Acids Res.* **13(16):** 5843-5856.

White, TJ, Arnheim, N and Erlich, HA (1989) The polymerase chain reaction. *Trends in Genetics* **5:** 185-189.

Williams, JF (1989) Optimization strategies for the polymerase chain reaction. *BioTechniques* **7(7):** 762-767.

Zelazny, B (1979) Distribution and spread of the cadang-cadang disease of coconut palm. *Acta Phytopath. Acad. Sci. Hung.* **14:** 115-126.

Zelazny, B and Niven, BS (1980) Duration of the stages of cadang-cadang disease of coconut palm. *Plant Disease* **64:** 841-842.

Zelazny, B and Pacumbaba, E (1982) Incidence of cadang-cadang of coconut palm in the Philippines. *Plant Dis.* **66:** 547-549.

Zelazny, B, Randles, JW, Boccardo, G and Imperial, JS (1982) The viroid nature of the cadang-cadang disease of coconut palm. *Scientia Filipinas* **2:** 46-63.

Zuker, M (1989) On finding all suboptimal foldings of an RNA molecule. *Science* **244:** 48-52.