D. Hanold and J. W. Randles
Waite Agricultural Research Institute, University of Adelaide
Glen Osmond, South Australia

Coconut Cadang-Cadang Disease and Its Viroid Agent

In the early 1930s a devastating epidemic of a lethal disease of coconut palm (*Cocos nucifera* L.) was reported from southern Luzon in the Philippines. It was appropriately named "cadang-cadang" disease from the word *gadan-gadan* of the local dialect meaning "dying" (13). Anecdotal reports indicate that the disease probably occurred a few years earlier in the Philippines, at about the time a somewhat similar epidemic was reported on the island of Guam. The disease in Guam was called "tinangaja" and was extremely destructive to the island's coconut population (2). Both diseases have now been shown to be caused by related viroids: coconut cadang-cadang viroid (CCCVd) and coconut tinangaja viroid (CTiVd).

Economic Importance

It has been estimated that over 30 million coconut palms have been killed by cadang-cadang since it was first recognized (22). Based on average yield and copra prices, the loss of production has been valued at about $80–$100 (U.S.) for each planting site occupied by an infected tree (13), an amount exceeding 2 months' salary for an unskilled worker in the Philippines. According to G. Persley (in a paper presented in 1989 to the Technical Advisory Committee of the Consultative Group on International Agricultural Research), at least 96% of the total world copra production is provided by smallholders. Because coconut is both an important subsistence and a major cash crop in many developing countries, cadang-cadang must be considered a serious economic threat (21).

Tinangaja disease is still prevalent on Guam. Its economic importance is less evident, however, because coconuts are not commercially produced there at present (2).

Symptoms and Host Range

Cadang-cadang develops slowly in palms and cannot be unequivocally identified on the basis of symptoms at a single observation. The disease progresses through three well-defined stages (13) (Fig. 1). In the early stage, nuts become rounded, with characteristic equatorial scarifications, and the first nonnecrotic, translucent, bright yellow leaf spots appear. In the mid stage, inflorescences become necrotic, nut production ceases, new frond production slows down, and leaf spots become larger and more frequent so that fronds begin to appear chlorotic from a distance. In the late stage, preceding death, leaf spots are almost confluent; the whole crown is distinctly yellowish or bronze-colored and very much reduced in size and number of fronds. The early stage lasts 2–4 years, the mid stage approximately 2 years, and the late stage about 5 years. The overall time from first symptoms to death of the tree is about 8 years for 22-year-old palms and about 16 years for 44-year-old palms (22). Usually, palms become naturally infected only after they have reached the age of flowering. In the rare cases where younger palms become infected, they are stunted and fail to produce inflorescences, although they survive well past the age of first flowering. Many cultivars and hybrids have been tested for susceptibility by inoculation in the Philippines, but none have shown any indication of immunity to the viroid. The only known variation in the symptoms of cadang-cadang is a more severe type of lamina reduction that occurs rarely in the field but has been observed in about 3% of mechanically inoculated coconut palms at the Albay Research Center in the Philippines. Parts of the fronds consist only of the midribs, thus showing a "brooming" syndrome (Fig. 2).

Tinangaja disease shows symptoms similar to those of cadang-cadang except that the affected trees bear spindle-shaped nuts with a reduced or absent kernel (2) (Fig. 3). There are a number of diseases of coconut palm with unknown etiology, some of which show abnormalities reminiscent of cadang-cadang or tinangaja, for example, narrow nuts as for Tatipaka disease and a decline as for Kerala wilt, both diseases occurring in India. Some of them have tested negative by gel electrophoresis (3) and solution molecular hybridization assay (12), but they need to be reevaluated by the latest molecular diagnostic methods for the presence of viroid.

Oil palm (*Elaeis guineensis* Jacq.) naturally infected or inoculated with CCCVd in the Philippines develops bright orange leaf spots that are larger and more numerous on the older fronds (Fig. 4), nut production ceases, and the tree eventually dies (14). Naturally infected buri palm (*Corypha elata* Roxb.) shows chlorotic leaf spots and stunting (14). Palm species successfully inoculated with CCCVd include oil palm, buri palm, betelnut palm (*Areca catechu* L.), golden cane palm (*Chrysalidocarpus lutescens* H. Wendl.), date palm (*Phoenix dactylifera* L.), royal palm (*Roystonea regia* (Kunth) Cook), and Manila palm (*Veitchia merrillii* (Becc.) Moore) (6,13).

Preliminary evidence suggests that several herbaceous monocotyledonous species growing near coconut palms infected with cadang-cadang (Fig. 5) occasionally contain viroidlike molecules with sequence similarity to CCCVd (J. M. B. Rodriguez, D. Hanold, and J. W.

Dr. Randles's address is: Department of Crop Protection, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia 5064.

Randles, *unpublished*). Such plants have no apparent symptoms. Isolation and characterization of these molecules and a comparison with CCCVd isolates is in progress.

Distribution, Epidemiology, and Control

The present distribution of cadang-cadang (Fig. 6) shows the northernmost boundary to be at the latitude of Manila and the southernmost at the latitude of Homonhon Island. The northern and southern boundaries are monitored annually. This is particularly important because of the proximity of the disease to the major coconut and oil palm growing area of Mindanao (16). Rates of spread vary such that some sites show little expansion, while others show active epidemics with outward movement of boundaries at about 0.5 km per year. Diseased palms are not clustered.

Little else is known about the epidemiology of cadang-cadang. The available data suggest that it may not be spread by any one specific route, but that it could be distributed by a variety of means (16). For example, the viroid can be detected in the husk (J. M. B. Rodriguez, *unpublished*) and embryo (D. Hanold, *unpublished*) of nuts and is seed-transmitted at the low rate of about one in 300 (18). It has been detected in purified pollen (D. Hanold, *unpublished*), and trials to determine whether transmission occurs via pollination are in progress at the Albay Research Center. Although many tests have failed to identify any insect vector (22; E. P. Pacumbaba, *unpublished*), it still seems possible that the viroid could be transmitted unspecifically by certain coleopterous insects through feeding wounds. The possibility that CCCVd can be transmitted by mechanical damage from cultural practices has yet to be adequately tested. Experimental plots have been set up in the Philippines with mechanically inoculated infector plants distributed in different arrays to determine the rate and pattern of spread of the viroid into the healthy population.

No control measures are known. Early attempts at control by eradication failed to prevent spread (22). As mentioned above, no resistant cultivars are available for replanting or as breeding material. No vector is known, so vector control is not an option. At present, the replacement of infected palms is the only practice that allows production to continue in affected areas, since the rate of spread in the new plantings is not influenced by the proximity of infected palms (12). Recently detected CCCVd-related viroids may be mild strains that could be used in mild strain protection, and this is an option for further study.

Molecular Characteristics of the Pathogen

It was reported in 1975 that viroidlike ribonucleic acid (RNA) was associated with cadang-cadang. This RNA was concentrated in a polyethylene glycol 6000 precipitate of leaf extracts, deproteinized, and identified by electrophoresis in polyacrylamide gels (PAGE) (11). The RNA was of low molecular weight, single-stranded (19), predominantly circular (17), and infectious (15). It was also found in naturally infected oil and buri palm (14), and a similar RNA was shown to be associated with tinangaja (3). These results, and the insensitivity of the disease to tetracycylines (15), were the first evidence that the disease had a viroid etiology.

Further analysis of the viroid on gels with a high resolving capacity showed that it comprises two monomeric "slow" and two monomeric "fast" forms (Fig. 7A), as well as dimeric forms of each monomer. Determination of their nucleotide sequences (5) (Fig. 7B) showed that the fast RNAs contain either 246 nucleotides or, by insertion of an additional cytosine residue at a specific site in the molecule, 247 nucleotides. The

Fig. 2. Reduction of lamina in an inoculated palm, giving the unusual "brooming" syndrome.

Fig. 1. (A) An area with a high incidence of cadang-cadang disease, showing trees in the early, mid, and late stages. (B) Leaflets from (left) healthy palm and (right) palm with late-stage disease showing nonnecrotic chlorotic spotting. (C) Nuts from (left) healthy palm and (right) diseased palm showing rounding, equatorial scarifications, and reduced husks. (D) Terminal necrosis of inflorescence and premature loss of male florets at early stage of disease.

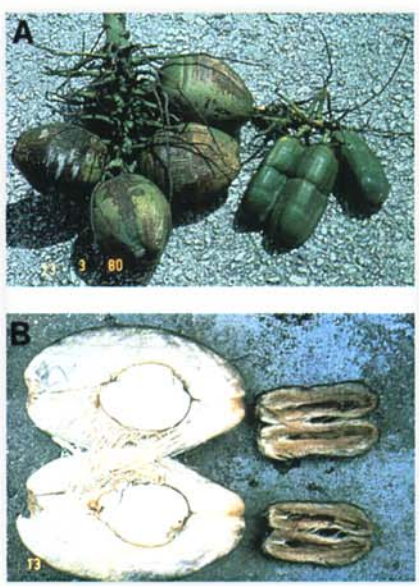


Fig. 3. Nut abnormalities associated with tinangaja disease: (A) (Left) Scarifications and (right) characteristic spindle shape. (B) Sections of nuts from (left) healthy and (right) diseased palm showing lack of kernel development. (Courtesy G. Boccardo)

slow RNAs of 296 and 297 nucleotides also differ by the addition of the cytosine, and the slow RNAs are directly derived from the corresponding fast forms by a duplication of the right-hand end of the molecule (Fig. 7).

In coconut palms, frond position is age-related, and an analysis of consecutive fronds in crowns of palms in the early, mid, and late stages of the disease showed that the four distinct molecular forms of the viroid occurred in a progression associated with the stage of the disease (7,13) (Fig. 7A). At the early stage, only the fast forms are found, but on subsequently emerging fronds the slow forms appear and eventually replace the fast forms as the disease proceeds through the mid to late stages. The forms of viroid detected in each frond remain the same as the frond ages and moves downward through the crown. It would be of interest to know whether duplication of the right-hand end of the viroid is involved in the transition of symptoms from the early to the mid and late stages, that is, to inflorescence necrosis and loss of nut production.

CCCVd has the central nucleotide sequence that is conserved among all viroids of the potato spindle tuber viroid group (9). Its physical properties and melting profile are similar to those of other viroids (20), with an intermediate structural form arising from annealing of complementary inverted sequences either side of the central conserved region. It is most closely related by size and nucleotide sequence to CTiVd, which has been shown to be 64% homologous with CCCVd (8). Compared with the other viroids, CCCVd has several unique features. As the 246 nucleotide form, it is the smallest known pathogen; with CTiVd, it is both the only viroid known to affect monocotyledonous plants and the only viroid that is lethal in a host plant; and its pattern of changing molecular forms has not been reported for any other viroid.

The infectivity of purified forms of CCCVd and their ability to induce disease (Fig. 8) has been demonstrated by high-pressure injection into folded leaf tissue (6,10,15), with the highest efficiency being achieved by injection close to the meristem of young sprouts. Increasing the concentration of RNA in the inoculum or increasing the number of injections raises the rate of successful inoculations. The 246/247 nucleotide form apparently has a higher specific infectivity than the others (10).

Fig. 4. Orange leaf spot development on fronds of oil palm inoculated with coconut cadang-cadang viroid; frond age increases from left to right.

Fig. 5. *Alpinia* sp. (foreground) associated with coconut palms infected with cadang-cadang. RNA related to coconut cadang-cadang viroid has been found occasionally in this and other monocotyledonous weeds.

Diagnosis

Because diagnosis of CCCVd by symptoms is unreliable, molecular diag-

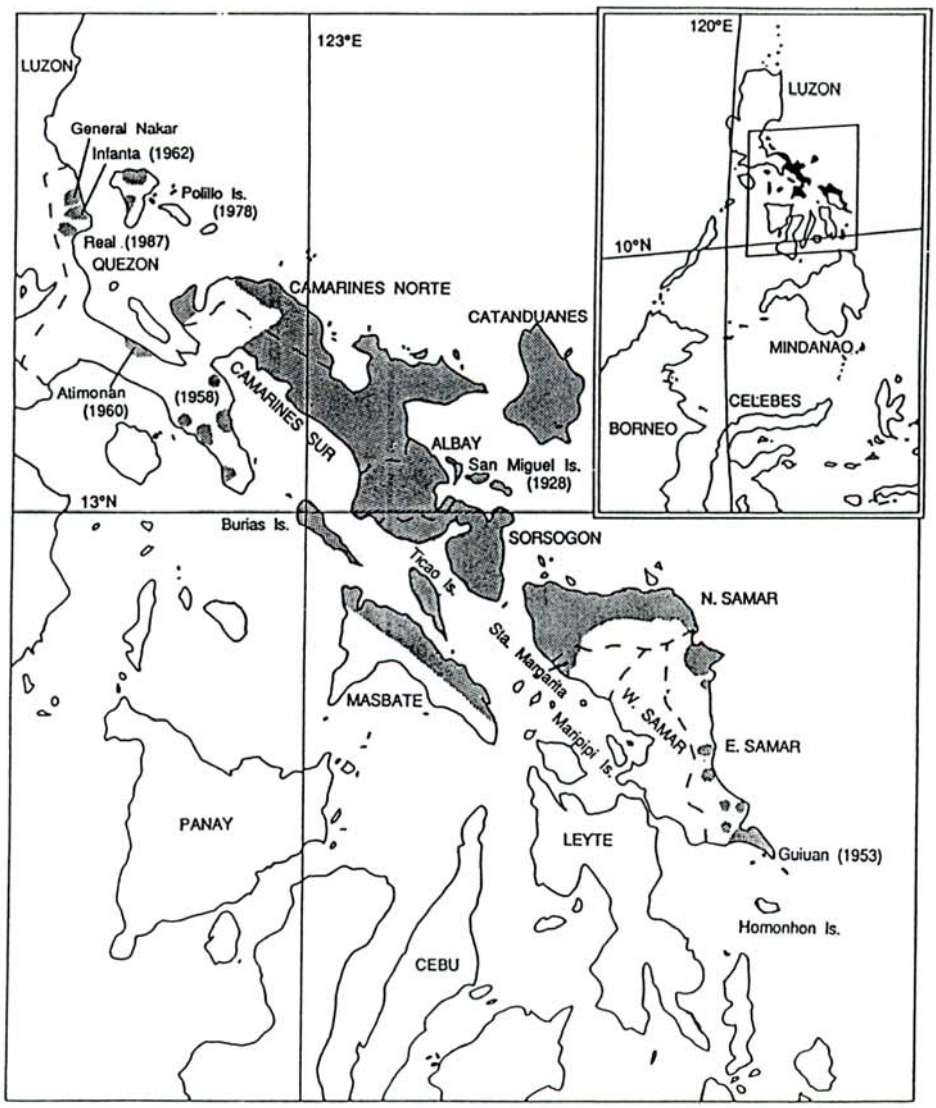


Fig. 6. Distribution of cadang-cadang disease (gray areas) in the Philippines.

nostic methods have been developed that rely on detection of the viroid in test samples.

When cellular nucleic acids purified from approximately 1 g of coconut tissue are analyzed on polyacrylamide gels, the molecular forms of CCCVd can be identified by their relative electrophoretic mobility. With use of the highly sensitive silver stain (6), CCCVd can be detected up to about 6 months before the appearance of initial symptoms. Normally, leaf material is used for routine analysis, but the viroid seems to be present in most host tissues. The method of diagnosis by gel electrophoresis has been successfully adapted for work in a mobile laboratory and is now the method of choice for epidemiological field surveys and for the yearly monitoring of disease boundaries in the Philippines.

CCCVd has been cloned (5) and can also be amplified by the polymerase chain reaction (PCR) (J. M. B. Rodriguez, J. W. Randles, and D. Hanold, *unpublished*). The clones and PCR product can be used as templates for synthesis of radioactively labeled complementary RNA or DNA probes. These are used in hybridization assays to detect nucleotide sequences similar to those of CCCVd (1). Extracts to be tested are applied to a supporting membrane, and the presence of viroid is detected by its specific hybridization with probe (Fig. 9A). The radioactive label causes darkening of exposed x-ray film, while samples without viroid show no signal. This diagnostic method is called "dot blot hybridization"; because it is much more sensitive than electrophoresis, viroid can be detected many months before symptoms appear.

The most conclusive results are obtained by a combination of these two methods in which electrophoretically separated nucleic acids are transferred to a membrane filter and then hybridized with radioactive probe (Fig. 9B). This "gel electroblot hybridization" method tests for both molecular size and structure, as well as sequence homology, and both one- and two-dimensional (Fig. 9C) gel electrophoresis (13) can be used. It is currently the most reliable and sensitive tool for finding CCCVd-related molecules in any type of host and tissue.

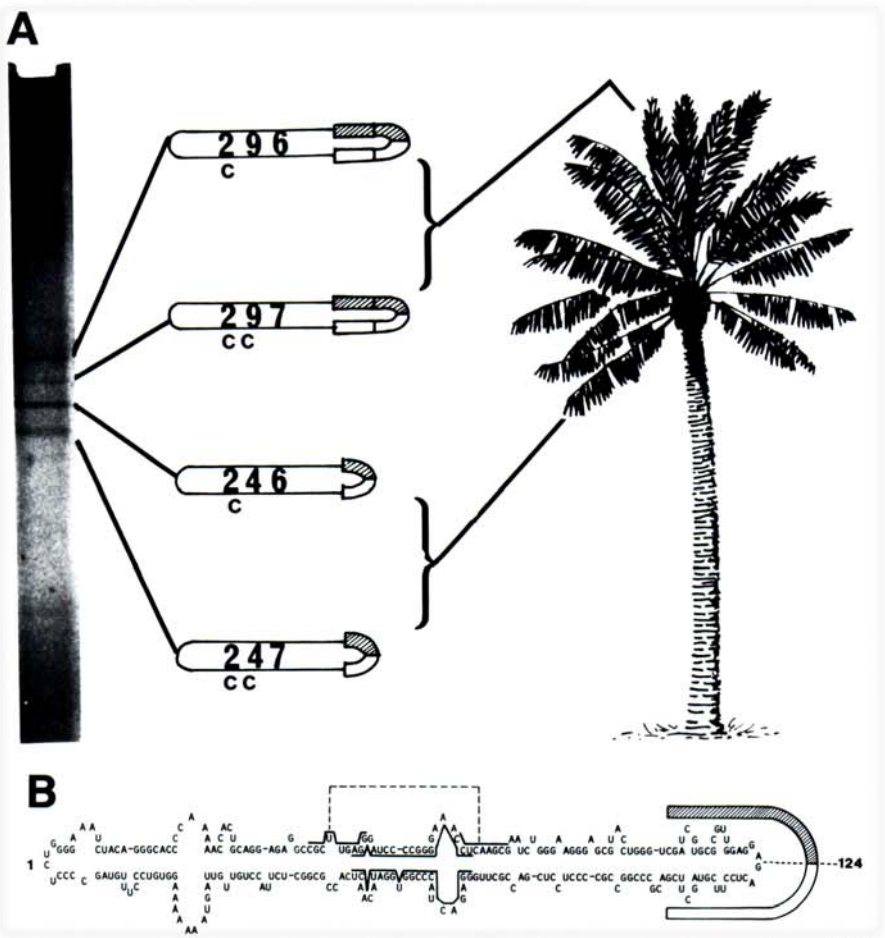


Fig. 7. (A) (Left) The four molecular forms of monomeric coconut cadang-cadang viroid separated by 20% polyacrylamide gel electrophoresis and (right) their distribution between older (infected first) and younger fronds of a diseased palm. (B) Sequence of the 247 nucleotide form of coconut cadang-cadang viroid. Deletion of a cytosine at position 197 produces the 246 form, and duplication of the right terminus (indicated by arc) produces the slow 296 and 297 forms. The internal bold lines mark the central conserved region, and the connected exterior lines mark the complementary inverted sequences.

CCCVd-related Sequences in New Locations

We have recently found viroidlike molecules related to CCCVd in several areas of the southwest Pacific (4). Electroblots of both one- and two-dimensional gels (Fig. 9C) have identified molecules of a mobility similar to that of CCCVd in oil palms in commercial plantations, in coconut palms, and in several herbaceous monocotyledons. The affected oil palms show symptoms resembling those in naturally infected oil palms (Fig. 10) in the Philippines, but the coconut palms are without the typical cadang-cadang syndrome (Fig. 11). The question as to whether the oil palm and coconut isolates are the same, as well as how closely they are related to CCCVd, must be evaluated by sequencing studies. It must be emphasized that no epidemic of cadang-cadang has been reported in these countries. Factors such as the viroid strain, host reaction, environment, or absence of an efficient putative vector may explain this. Identification of the factors preventing the development of a serious outbreak may eventually help in the development of control measures for CCCVd in the Philippines. For example, certain environmental requirements for viroid transmission may be essential or mild strains of CCCVd may be identified that could be used for "mild strain protection" when present in a host plant before challenge by the severe strain.

Conclusions

The viroid etiology of cadang-cadang disease of coconut palm in the Philip-

Fig. 8. Coconut palm mechanically inoculated with coconut cadang-cadang viroid (foreground) showing yellowing, stunting, tapered stem, reduced crown, and no nuts; (background) healthy palm of same age.

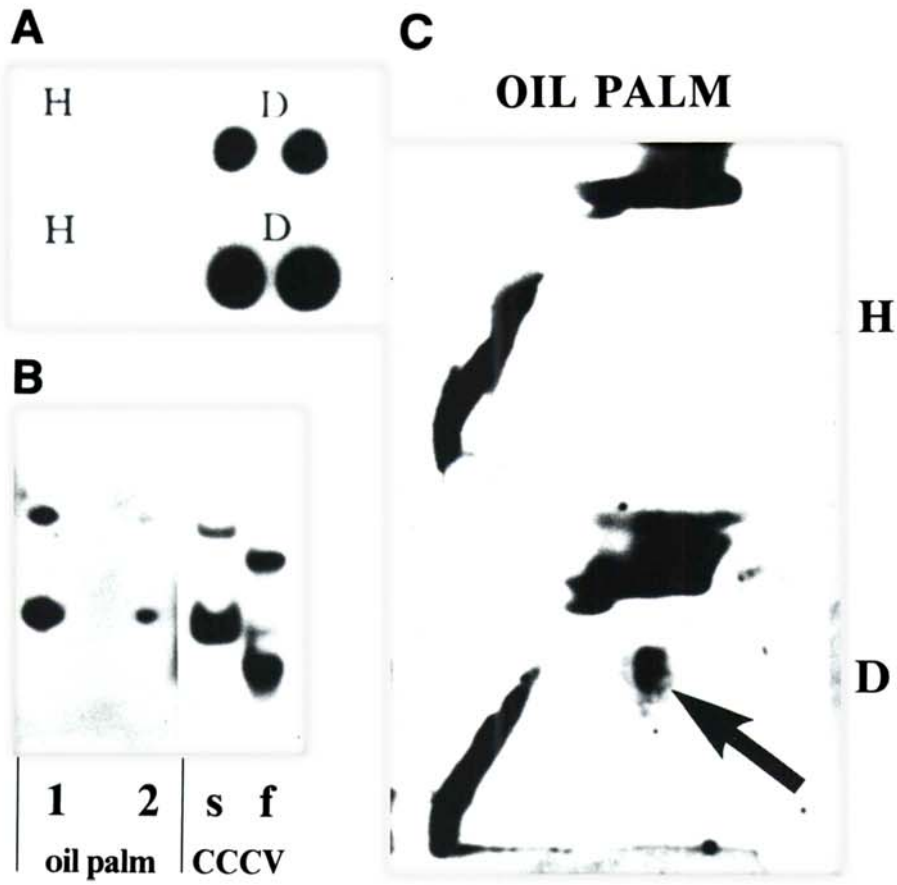


Fig. 9. Diagnosis of coconut cadang-cadang viroid: (A) Dot blot hybridization assay; H = healthy control, D = diseased coconut palm. (B) Electroblot hybridization assay showing coconut cadang-cadang viroid in a naturally infected oil palm; s = slow (296/7 nucleotides) and f = fast (246/7) forms of marker coconut cadang-cadang viroid, both showing monomers (lower major bands) and dimers (upper minor bands). (C) Two-dimensional polyacrylamide gel electrophoresis in association with an electroblot. (Top) Healthy palm; (bottom) coconut cadang-cadang viroid-related sequences in a naturally infected oil palm from the South Pacific; arrow indicates viroid-like nucleic acid that, because of its structure, moves at a different rate from that of other nucleic acids in the sample. Some nonspecific binding of probe in regions other than the viroidlike RNA is due to low-stringency washing conditions after hybridization in this experiment.

Fig. 10. Orange spotting of oil palm in a commercial plantation: (A) Whole crown. (B) Increasing size and density of spots on fronds of (left to right) increasing age.

Fig. 11. A coconut palm from the southwest Pacific containing coconut cadang-cadang viroid-related sequences. It does not show the typical syndrome, but some necrosis of inflorescences and a tendency toward spindle-shaped nuts, as for tinangaja, are apparent.

pines has been established, but no control measures are available and the disease continues to spread. Our recent identification of viroidlike sequences related to CCCVd in the Pacific outside the known cadang-cadang area raises a number of new questions about the origins, epidemiology, and pathogenicity of these CCCVd-related sequences. We consider that until more is known about the epidemiology of these viroidlike molecules, an embargo should be placed on the uncontrolled movement of germ plasm between countries. This is particularly important because the viroid infections appear to be latent and because their pathogenicity in new areas is unpredictable.

Current breeding practice is to produce hybrid seednuts of coconut or oil palm in seed gardens where the local or introduced female parent is established and pollen is either produced nearby or obtained from another country. All germ plasm used for this purpose must be assayed by means of the highly sensitive diagnostic tests now available, and elite certified planting material must be identified.

It is especially important that embryo cultures of coconut palm and tissue cultures of oil palm be derived only from viroid-tested material. The consequences of failure to ensure pathogen freedom with such multiplication procedures would result in very high levels of infection of clonally propagated material and the introduction of viroid to new areas and countries.

Acknowledgments

This paper is dedicated to W. C. Price, whose persistence and enthusiasm established the basis for this work. We are grateful to the Philippine Coconut Authority, the United Nations' Food and Agriculture Organization (1973–1984), and the Australian Centre for International Agricultural Research (from 1984) for support of research on the viroid aspects of cadang-cadang.

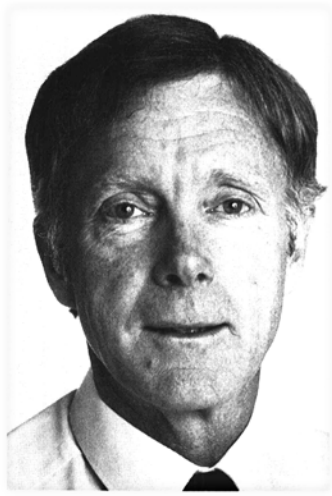
Literature Cited

1. Barker, J. M., McInnes, J. L., Murphy, P. J., and Symons, R. H. 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.
2. Boccardo, G. 1985. Viroid etiology of tinangaja and its relationship with cadang-cadang disease of coconut. Pages 75-99 in: Subviral Pathogens of Plants and Animals: Viroids and Prions. K. Maramorosch and J. J. McKelvey, Jr., eds. Academic Press, New York.
3. Boccardo, G., Beaver, R. G., Randles, J. W., and Imperial, J. S. 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 71:1104-1107.
4. Hanold, D., and Randles, J. W. 1989. Cadang-cadang-like viroid in oil palm in the Solomon Islands. Plant Dis. 73:183.
5. Haseloff, J., Mohamed, N. A., and Symons, R. H. 1982. Viroid RNAs of cadang-cadang disease of coconuts. Nature 299:316-321.
6. Imperial, J. S., Bautista, R. M., and Randles, J. W. 1985. Transmission of the coconut cadang-cadang viroid to six species of palm by inoculation with nucleic acid extracts. Plant Pathol. 34:391-401.
7. Imperial, J. S., and Rodriguez, M. J. B. 1983. Variation in the coconut cadang-cadang viroid: Evidence for single-base additions with disease progress. Philipp. J. Crop Sci. 8:87-91.
8. Keese, P., Osorio-Keese, M. E., and Symons, R. H. 1988. Coconut tinangaja viroid: Sequence homology with coconut cadang-cadang viroid and other potato spindle tuber viroid related RNAs. Virology 162:508-510.
9. Keese, P., and Symons, R. H. 1987. Physical-chemical properties: Molecular structure (primary and secondary). Pages 37-62 in: The Viroids. T. O. Diener, ed. Plenum Press, New York.
10. Mohamed, N. A., Bautista, R., Buenaflor, G., and Imperial, J. S. 1985. Purification and infectivity of the coconut cadang-cadang viroid. Phytopathology 75:79-83.
11. Randles, J. W. 1975. Association of two ribonucleic acid species with cadang-cadang disease of coconut palm. Phytopathology 65:163-167.
12. Randles, J. W. 1985. Coconut cadang-cadang viroid. Pages 39-74 in: Subviral Pathogens of Plants and Animals: Viroids and Prions. K. Maramorosch and J. J. McKelvey, Jr., eds. Academic Press, New York.
13. Randles, J. W. 1987. Coconut cadang-cadang. Pages 265-277 in: The Viroids. T. O. Diener, ed. Plenum Press, New York.
14. Randles, J. W., Boccardo, G., and Imperial, J. S. 1980. Detection of the cadang-cadang RNA in African oil palm and buri palm. Phytopathology 70:185-189.
15. Randles, J. W., Boccardo, G., Retuerma, M. L., and Rillo, E. S. 1977. Transmission of the RNA species associated with cadang-cadang of coconut palm, and insensitivity of the disease to antibiotics. Phytopathology 67:1211-1216.
16. Randles, J. W., Hanold, D., Pacumbaba, E. P., and Rodriguez, M. J. B. 1991. Cadang-cadang disease of coconut palm. In: Plant Diseases of International Importance. A. N. Mukhopadhyay, J. Kumar, H. S. Chaube, and U. S. Singh, eds. Prentice Hall, Englewood Cliffs, NJ. In press.
17. Randles, J. W., and Hatta, T. 1979. Circularity of the ribonucleic acids associated with cadang-cadang disease. Virology 96:47-53.
18. Randles, J. W., and Imperial, J. S. 1984. Coconut cadang-cadang viroid. No. 287 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, Surrey, England.
19. Randles, J. W., Rillo, E. P., and Diener, T. O. 1976. The viroid-like structure and cellular location of anomalous RNA associated with the cadang-cadang disease. Virology 74:128-139.
20. Randles, J. W., 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. Nucleic Acids Res. 10:5569-5586.
21. Thurston, H. D. 1973. Threatening plant diseases. Annu. Rev. Phytopathol. 11:27-52.
22. Zelazny, B., Randles, J. W., Boccardo, G., and Imperial, J. S. 1982. The viroid nature of the cadang-cadang disease of coconut palm. Scientia Filipinas 2:45-63.

D. Hanold

Dr. Hanold received her degree of Dipl. Biol. in microbiology from the University of Basel, Switzerland, working under the supervision of W. Arber. Her Ph.D. degree in 1982 was also gained at the University of Basel in the field of plant cell and molecular biology, with T. Hohn, B. Hohn, I. Potrykus, and W. Arber. She has since worked at the Swiss Cancer Research Institute, Lausanne, on virus replication, and at the Australian National University, Canberra, on molecular aspects of nitrogen fixation. In 1985, she joined the Waite Agricultural Research Institute, University of Adelaide, to work on the molecular biology of plant viruses, with a major interest in viroids.



J. W. Randles

Dr. Randles gained B.Ag.Sc. (Hons.) and M.Ag.Sc. degrees from the University of Adelaide, specializing in plant pathology and virology. After employment with the South Australian Department of Agriculture, he studied under R. E. F. Matthews at the University of Auckland for his Ph.D. degree. In 1969, he was appointed to the Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, where he is currently a reader. His special interests are viroids and characterization and epidemiology of plant viruses. He received the Lee M. Hutchins Award of the American Phytopathological Society in 1982.