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Localisation of coconut foliar decay virus in coconut palm

By J. W. RANDLES, D. C. MILLER, J. P. MORIN*, W. ROHDE** and D. HANOLD

Department of Crop Protection, Waite Agricultural Research Institute, University of Adelaide, South Australia
**Saraoutou Experiment Station, Santo, Vanuatu*
***Max-Planck-Institut für Züchtungsforschung, D-5000, Köln 30, Germany*

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Summary

Coconut foliar decay virus (CFDV) occurs at a very low concentration in coconut palm. A 1203 nucleotide segment of the sequenced encapsidated circular single-stranded 1291 nucleotide CFDV-DNA has been amplified and transcribed for use as a ^{32}P cDNA probe for the virus. A rapid method for the extraction of DNA from coconut palm has been devised for a dot-blot hybridisation assay using this probe. An alternative non-radioactive probe has also been developed for future use in CFDV diagnosis.

CFDV-DNA was shown to be distributed unevenly in a range of infected palms, necessitating the use of multiple sampling to reliably detect infection in diagnostic tests. Viral DNA was detected in symptomatic and asymptomatic palms of both high and low susceptibility, in disease-free tolerant cultivars, and in palms in remission from disease. Within the same palm, detectability of viral DNA varied little within leaflets, but varied more within and between fronds. CFDV-DNA was detected 6–8 months after insect-mediated inoculation, and symptoms generally appeared after another 1–4 months.

In situ hybridisation of rachis tissue showed localisation of DNA within the phloem, but its distribution in the phloem was uneven. CFDV-DNA was detected in tissue adjacent to and within necrotic zones which develop into the petiolar lesions associated with the disease-specific collapse of fronds.

Virus was detected in the body of the insect vector, and, where its distribution could be resolved, in the abdomen rather than the head.

Key words: Coconut foliar decay virus, hybridisation assay, ^{32}P-cDNA, DIG-cRNA, phloem, *Myndus*, PCR

Introduction

Coconut foliar decay (CFD) is a lethal disease of introduced coconut palms in Vanuatu (Calvez, Julia & de Nuce, 1985; Randles, Julia, Calvez & Dollet, 1986). Local dwarf and tall coconut varieties do not show the disease, whereas introduced susceptible palms are affected. These initially show yellowing of some of the leaflets on a frond 7 to 11 positions down from the unopened spear leaf, followed by more general yellowing of this and adjacent fronds. These fronds simultaneously develop a lateral necrosis near the base of the petiole and eventually collapse from a position near the necrosis, so that they hang down through

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the canopy. Progression of this process up and down the crown leads to death (Randles *et al.*, 1986). The period from symptoms to death takes 1 to 2 years depending on the susceptibility of the cultivar.

CFD is transmitted by *Myndus taffini* (Julia, 1982). The minimum inoculation time is about one hour, and infectivity is retained for 2–3 days (Julia, Dollet, Randles & Calvez, 1985), but other parameters such as the acquisition threshold and the mode of transmission are not known.

The association of 1.3 kb single-stranded circular DNA with diseased palms (Randles *et al.*, 1986; Randles, Hanold & Julia, 1987) and the detection of 20 nm icosahedral particles associated with the DNA (Randles & Hanold, 1989) showed that CFD was caused by a novel type of virus (CFDV). Structurally, CFDV most closely resembles porcine circovirus (Tischer, Gelderblom, Vettermann & Koch, 1982), subterranean clover stunt virus (Chu & Helms, 1988) and banana bunchy top virus (Thomas & Dietzgen, 1991; Harding, Burns & Dale, 1991), but is distinct from all of these.

Identification of disease by symptoms alone is unreliable, and so CFDV-DNA has been cloned and used for virus diagnosis (Hanold, Langridge & Randles, 1988). Sequencing (Rohde, Randles, Langridge & Hanold, 1990) of a complete 1291 nucleotide CFDV-DNA has identified a novel arrangement of putative open reading frames, one of which has replicase motifs.

This paper reports the development of a rapid and sensitive procedure for identifying CFDV in coconut tissue, using radioactive and non-radioactive probes complementary to the unique CFDV-DNA. It describes the distribution of CFDV in relation to disease symptoms in palms, and shows that the virus is associated with phloem of the palm and the abdomen of the insect vector.

Materials and Methods

Experimental plants

The trees used were from a range of lines of *Cocos nucifera* (Table 1) grown at the Saraoutou Experiment Station, Espiritu Santo, Vanuatu. Diseased trees were infected either artificially in an insect cage containing *Myndus taffini* collected from diseased trees in the field, or naturally (Julia, 1982) and selected according to their stage of disease development and symptom severity (Calvez *et al.*, 1985).

Sampling of tissue

Samples were collected from leaflets, rachis, spathes, inflorescences, nuts, trunk (using a 2 cm diameter augur) and both primary and secondary roots. To determine the distribution of CFDV-DNA within fronds, samples were collected from leaflets and rachis at the base, first quarter, middle, third quarter and tip of selected fronds. The leaflet sample was taken midway along the leaflet; a rachis sample was collected from the frond at the base of this leaflet. The leaflet from the middle of the frond was also sampled at the tip and base. Embryos were excised from mature nuts. Pollen was extracted by removing the anthers from unopened male flowers, placing them in 50 ml of 10 g/litre sodium dodecyl sulphate in a beaker until they were level with the top of the liquid, then squeezing them gently with the bottom of a small bottle to burst the anthers. Anthers were strained off with 2 mm nylon mesh, and the pollen was washed twice by allowing it to settle to the bottom of a measuring cylinder containing 50 ml of 1 g/litre SDS.

The slurry containing the pollen was examined with a microscope to ensure that it was free of anther fragments, then the pollen was sedimented at 1000 *g* for 1 min.

Extraction procedure

Tissue samples were extracted by crushing in one volume (w/v) of TNET (50 mM Tris-HCl, pH 7.2, 100 mM sodium acetate, 10 mM Na_2 EDTA, 10 ml/litre mono-thioglycerol). Crushing was generally done by placing 1 g of tissue and 1 ml of TNET in a small, heavy duty plastic bag, and using a hammer to crush and macerate the tissue (Randles, Hanold, Pacumbaba & Rodriguez, 1992). An aliquot of 0.3 ml of the juice was mixed with 0.3 ml of 1 N potassium hydroxide and incubated for 2 h at 25°C. To this was added 0.3 ml of 90% phenol (containing 1 g/litre 8-hydroxy-quinoline) and 0.3 ml chloroform, and the mixture was mixed vigorously for 1 min. It was then centrifuged at 10 000 *g* for 10 min, and 0.3 ml of the supernatant was mixed with three volumes of ethanol. The ethanol precipitate was collected by centrifugation, washed with ethanol, dried, and resuspended in 20 μl TE (10 mM Tris HCl, pH 7.5, 0.1 mM EDTA). The solution was clarified by centrifugation before use.

Compacted pollen was mixed with 1 volume (v/v) of TNET, placed in a mortar, frozen, then ground with a pestle at room temperature until microscopic examination showed that essentially all pollen grains had lost their nuclei. The samples were then extracted with 0.1 vol. 100 g/litre SDS, and 0.5 vol. each of phenol and chloroform by agitation for 1 h at 25°C. After centrifugation, nucleic acids in the supernatant were precipitated with three volumes of ethanol, and the pellet was dried.

The anther component was washed twice in 0.1% SDS to remove most adhering pollen grains. Duplicate samples of 1 g were mixed with 1 ml of TNET, crushed and extracted by mixing with one volume of 1 N potassium hydroxide, and incubating for 2 h at 25°C. A phenol and chloroform extraction, and ethanol precipitation, was done as above.

Synthesis of ^{32}P cDNA

Double-stranded DNA of 1203 bp representing 93% of the sequence of encapsidated 1291 nucleotide CFDV-DNA was amplified by the polymerase chain reaction (Rohde *et al.*, 1990). The primer for complementary sense DNA was 5′ GGGACTCCGTGCTCGA-GAAGCACCCGTT 3′, that for virion sense DNA was 5′ CCGAAGATGCGTTGTA-CACGGAGCTTCGG 3′. Reaction mixtures contained 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, 100 μg/ml gelatin, 0.2 mM each of dATP, dCTP, dGTP and TTP, ss CFDV circular DNA template at *c.* 4 ng/ml, primers each at *c.* 120 ng/ml, and *Taq* polymerase at 30 units/ml (Perkin Elmer Cetus, GeneAMP™ kit). Reaction mixtures of 25–50 μl in siliconised 1.5 ml tubes were heated to 96°C for 3 min, cooled to 72°C, the *Taq* polymerase was added, and 25 μl of light mineral oil (Sigma, St. Louis, USA) was added to seal the surface. A Hybaid IHB 2024 temperature cycler was used to provide 40 cycles comprising 55°C for 45 s for primer annealing, 72°C for 3 min for extension, and 93°C for 45 s for product denaturation. The final extension step was for 15 min, and products were separated by electrophoresis on 12 g/litre agarose gels. Bands stained with ethidium bromide were excised and recovered by electro-elution (Randles & Rohde, 1990). ^{32}P cDNA was prepared from amplified DNA which had been denatured at 100°C for 5 min. The reaction mix (30 μl) contained 1–2 μg of the DNA, 7.5 μg hexa-deoxynucleotide primers, 7 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 50 μM each of dATP, dGTP and TTP, 1.1 MBq of ^{32}P dCTP (111 TBq/mM) and three units of Klenow DNA polymerase (Boehringer Mannheim) and was incubated at 25°C for 16 h. The reaction mix was diluted with 30 μl TE and the cDNA fractionated on a Sephadex G50 column.

In situ *hybridisation with coconut tissue*

Leaf and rachis samples were fixed in 1% (w/v) glutaraldehyde buffered with 50 mM Tris-Cl, pH 7.2 and stored for 3–4 wk at 4°C. Thin sections were cut from the rachis samples with a sledge microtome (Leitz, Wetzlar) and stored in the same fixative at 4°C. Sections were treated with 0.25 N potassium hydroxide for 20–30 min at 22°C, neutralised in 100 mM Tris-HCl, pH 7 for 15–30 min, then incubated at 42°C for 5–24 h in prehybridisation buffer according to Hanold & Randles (1991). For hybridisation, ^{32}P CFDV cDNA in 500 ml/litre formamide was heated at 100°C for 2 min, and mixed with hybridisation buffer to give a final radioactivity of about 1×10^6 cpm/ml (Hanold & Randles, 1991). Sections were incubated at 42°C for 70 h. They were then washed in 1 × SSC (0.15 M NaCl, 0.015 M Na citrate) with 1 g/litre SDS for 1 min at 22°C, for 4 h at 65°C, then in 0.1 × SSC, 1 g/litre SDS for 1 h at 65°C after which the washing temperature was reduced to 48°C and washing continued for 16 h. Moist sections were covered with polythene film and autoradiographed at room temperature.

Rachis samples which were positive in the above assay were selected for higher resolution *in situ* autoradiography. Sections were cut with a freezing stage microtome. These were subjected to hybridisation as above. Microscope slides were cleaned with 2 N potassium hydroxide, and sections were placed within 25 mm of one end. Molten 150 g/litre gelatin was placed under and over the section, a siliconised coverslip was placed over the section so as to exclude air and to ensure that surface irregularities in the section were filled with gelatin. The slide was placed at 4°C to set the gelatin. The coverslip was removed immediately before coating the section with liquid photographic emulsion as follows. In the dark room, strips of emulsion (Ilford K5) were made up to a volume of 7 ml, melted by incubation at 45°C for 1 h, then mixed gently with 7 ml of water at 45°C for 3 h. Sections were coated by dipping slides for 5 s and wiping off the excess emulsion. Slides were drained at an angle of 30°C, airdried for 2 h, then stored in a container with activated silica gel at 4°C for at least 8 days. Emulsion was developed for 2 min (Kodak liquid x-ray developer, Type 2) washed, fixed, washed and dried. All dark room operations were done with a Kodak No. 2 safelight. At this stage, silver was observed macroscopically over parts of the tissue. Photomicrographs were made of the distribution of silver grains at instrument magnifications of 200 to 800×.

In situ *hybridisation with* Myndus taffini

Adult *Myndus taffini* were collected from coconut leaflets in the field and killed by freezing. They were fixed in a 3% (w/v) glutaraldehyde (buffered with 0.1 M phosphate buffer, pH 7, containing 0.1 g/litre sodium dodecyl sulphate) for 4 days at 5°C. They were rinsed then stored in 0.1 M phosphate buffer at 5°C.

Insects were cut longitudinally with a steel blade, the halves were each treated with 0.25 N potassium hydroxide at 25°C for 30 min, then incubated in prehybridisation buffer and finally hybridised with ^{32}P cDNA as described above for plant tissue. The halves were then washed in 1 × SSC, 1 g/litre SDS, then 0.1 × SSC, 1 g/litre SDS, each for 1 h at 65°C. Three final washes, each of 24 h, were done in 0.1 × SSC, 1 g/litre SDS, at 65°C until the radioactivity of the wash reached a constant level of about 100 cpm/ml. Separate halves were then placed on adhesive coated paper, covered with polythene film, and autoradiographed for 8 days at −70°C, using an intensifying screen.

Dot hybridisation

Plant nucleic acid samples of 1 μl were applied as dots to nylon membrane (Zeta probe, Bio-Rad), baked at 80°C for 2 h, prehybridised and hybridised as described above, except

that the incubation times for each step were 16–24 h. Membranes were washed in 1 × SSC, 1 g/litre SDS, then 0.1 × SSC, 1 g/litre SDS, both for 1 h at 65°C. They were autoradiographed at −70°C using an intensifying screen.

Non-radioactive hybridisation assay for CFDV-DNA

Digoxygenin (DIG) labelled cRNA of negative orientation was transcribed from a pSP65 vector containing the full sequence of CFDV using SP6 RNA polymerase and DIG-αUTP, according to the directions of the manufacturers (Boehringer Mannheim). Nylon membranes were prepared with samples as described above and hybridisation was done at 42°C with the blocking agent and buffer supplied by the manufacturer, including *c*. 50 ng/ml of DIG-cRNA. The membranes were washed twice for 15 min in 2 × SSC with 1 g/litre SDS at 68°C. Probe was allowed to bind anti-DIG-alkaline phosphatase conjugated antibody (1:2000 dilution) for 30 min at 25°C. The membrane was washed and equilibrated in the appropriate buffers, then incubated with AMPPD^R substrate (100 μg/ml) for 5 min. The membrane was drained, sealed in a plastic bag, preincubated for 15 min at 37°C, then exposed to x-ray film for a range of times to detect the chemiluminescent signal associated with positive samples.

Coconut cultivars

The abbreviations for coconut cultivars are as follows: BGD = Brazilian Green Dwarf; RLT = Rennell Tall; MRD = Malayan Red Dwarf; PGD = Philippine Green Dwarf; CRD = Cameroon Red Dwarf; NLD = Niu Leka Dwarf; VRD = Vanuatu Red Dwarf; VTT = Vanuatu Tall; TGD = Thailand Green Dwarf; and WAT = West African Tall.

Results

Assays for CFDV-DNA

Reliable detection of CFDV-DNA by dot-blot hybridisation with ^{32}P was dependent on using a 0.5 N potassium hydroxide incubation step at 25°C for extracting the crushed leaf tissue. Very weak or no signals were frequently obtained from infected samples when incubation in alkali was omitted. The signal was also inexplicably lost if the alkali was neutralised with hydrochloric acid before phenol-chloroform extraction. The use of nylon

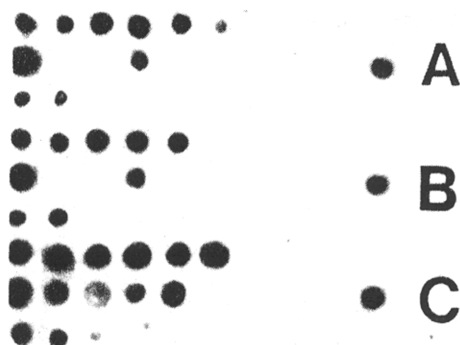

Fig. 1. Detection of CFDV-DNA with DIG-cRNA and AMPPD in nucleic acid extracts which were either untreated (C), or incubated with 0.5 N NaOH for 2 h at either 25°C (A) or 37°C (B). From top left in each group, samples 1–5, 11, 14 are from infected palms, 20–24 are standards in a 5 fold dilution series. The remainder are from healthy plants. Note the false positives in 6, 12, 13, 15 for A and C. Results from B were confirmed with ^{32}P cDNA.

Table 1. *Distribution of CFDV-DNA in a range of coconut palms, shown in relation to cultivar or hybrid, duration of disease, and severity of host reaction*

Disease susceptibility of cultivar	cv.	mode of inoc	Age (yr) at first symptoms	at assay	DISEASE STATUS OF PALM AT ASSAY	H	R2*	R1*	T	F27	F25	F23	F21	F19	F17	F15	F13	F11	F9	F7	F5	F3	F1
HIGH	BGD			5	ASYMPTOMATIC		0	0	0							0	0	0	0	0	0	0	0
	RLT	N		11		0	1	1	1					0	0	0	0	0	0	0	0	1	1
	RLT	N		11		0	1	1	1								0	1	1	0	0	0	0
	BGD	N	4.7	5	SYMPTOMATIC		1	1	1										1	1	1	1	1
	MRD	N	5.6	6			0		0							1			1		1		
	MRD	N	5.6	6			0		0		0	0	0	0	0	0	1	1	1	1	1	0	1
	PGD	N	7.4	8			0		0	0	0	D	D	D	D	D	1	1	1	1	1	1	1
	PGD	N	7.7	8			1		1		0	1	1	1	1	1	1	1	1	1	1	1	1
	CRD × NLD	N	15.7	16		1	1	0	1									1	1	1	1	1	1
	CRD × NLD	N	15.4	16		1	1	1	1								1	1	1	1	1	1	1
	RLT	N	8	11		0	1	1	1								0	0	0	0	0	0	1
	RLT	N	9	11			1	1	1							1	1	1	1	1	1	1	1
					REMISSION for																		
	MRD	I	1	6	4y	0	0	0	1							0	1		1	1	1	1	1
	MRD	I	1	7	5y	1	1	0	1					0	0	0	0	0	1	0	1	1	0
	RLT	N	7	11	3y	0	1	1	1						0	1	0	1	1	1	1	1	1
	RLT	N	7	11	3y	0	1	1	1						1	1	1	1	1	1	1	1	1

LOW	VRD	N		9	ASYMPTOMATIC	0	1	0	1					0	0	0	0	C	0	0	0	0
	VRD	N		9		0	1	1	1					0	0	0	0	0	0	1	1	1
	RLT × VTT			9		0	0	0	0					0	0	0	0	0	0	0	0	0
	RLT × VTT	N		9		0	0	1	0				0	0	0	0	1	0	1	0	0	1
	RLT × VTT	N		9		0	0	0	1				1	1	1	1	1	1	1	1	0	1
	BGD × VTT	N	7	9	SYMPTOMATIC		1	1	0							1	D	1	1	1	1	1
	BGD × VTT	I	2	9	REMISSION for 5y	1	1	1	1				1	1	1	1		1	1	1	1	1
TOLERANT	VTT	N		11	ASYMPTOMATIC	0	0	0					1	1	1	1	1	1	1	1	0	0
	VTT	N		11		1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	VTT	N		11		1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0
	VTT	I		3			1		0									0	0	0	0	0
	VTT	I		3			1		0									1	0	0	0	0
	VTT	N	10	11	SYMPTOMATIC		0	0									1	1	1	1	1	1

0 = negative
1 = positive
F = frond
T = trunk
R1* = primary root
R2* = secondary root
H = husk
D = dead
I = artificial inoculation
N = natural field inoculation

as a support membrane was essential as nitrocellulose was frequently denatured by the samples during the baking fixation step. Charged nylon (Zeta probe) was superior in signal strength to uncharged nylon (Hybond N, Amersham). All results reported in this paper are based on this assay.

In non-radioactive tests using DIG labelled cRNA, in combination with anti-DIG conjugated antibody and a phosphatase detection assay using AMPPD, false positives were detected as a result of endogenous phosphatase activity in some samples, despite the use of an incubation in 0.5 sodium hydroxide at 25°C. This endogenous enzyme activity was removed, however, by incubating leaf extracts in 0.5 sodium hydroxide for 2 h at 37°C (Fig. 1). This alternative assay may be useful in the future.

The sensitivity of both assays for CFDV DNA was in the picogram range with the AMPPD system detecting CFDV-DNA in a much shorter exposure time (minutes) than with ^{32}P labelled cDNA (autoradiography for 6–72 h).

Detection of CFDV-DNA in relation to symptom expression

The coconut palm collection at Saraoutou has been classified according to susceptibility to coconut foliar decay disease (Calvez *et al.*, 1985). In this study, palm selections and hybrids were separated (see Table 1) into classes showing high susceptibility, low susceptibility and tolerance. Highly susceptible lines show typical symptoms leading to premature death (Randles *et al.*, 1986). Lines of low susceptibility show mild foliar symptoms and a small number of these populations may die. Tolerant lines do not show the disease at all, even when grown in high incidence areas. In addition to this classification, remission of disease has been observed in some palms of both tall and dwarf cultivars which were formerly symptomatic (Anon., 1990). Remission was initially described in some palms which had been inoculated at the seedling stage by exposure to large numbers of the vector *Myndus taffini* collected from the field. Symptoms appeared as for the rest of the population, but then disappeared 1–2 years later. Such plants, even though they were exposed to high infection pressure by planting in an area of high disease incidence and vector activity, then remainded disease free. These palms are still in remission after observation for up to 5 years (Table 1). Remission has also been observed in field infected palms (e.g. the RLT palms in Table 1). CFDV-DNA could be detected in palms from the high and low susceptibility as well as the tolerant groups, and also in palms in remission (Table 1). In symptomatic palms, leaf tissue from fronds with the symptoms, or those above, usually contained detectable CFDV-DNA. In palms of tolerant cultivars and infected asymptomatic palms of susceptible cultivars, few or no fronds were positive. In some of these trees, such as one of the asymptomatic tolerant VRD palms (Table 1), only the trunk or the roots indicated the presence of virus DNA. Palms in remission showed essentially the same CFDV-DNA distribution as symptomatic palms.

The VTT population was shown to contain CFDV DNA and thus is not immune to infection although it is generally asymptomatic. The one symptomatic palm included with VTT in Table 1 may have arisen from cross-pollination of VTT with another variety, or the symptoms may have been caused by other factors.

Artificially inoculated MRD seedlings first showed detectable CFDV-DNA at 6 to 8 months post-inoculation, with symptoms generally appearing after another 1 to 4 months (Table 2).

Distribution in palm tissue

The distribution of CFDV-DNA in individual palms was uneven, and no particular tissue of the infected palms was identified as always having detectable levels of CFDV-DNA.

Table 2. *Times to first detection of CFDV-DNA and appearance of symptoms for inoculated MRD seedlings*

Months after inoculation[a]		
CFDV-DNA	Symptoms	Number of seedlings
6	asymptomatic	1
6	7	1
6	9	13
6	10	1
8	asymptomatic	4
8	9	2
8	11	2
8	12	4

[a] Observations were made for 14 months.

Table 3 shows the frequency of detection of CFDV-DNA by dot hybridisation assay in various tissues. CFDV-DNA could be detected in both leaf and rachis tissue. However, it was not necessarily detected in both leaf and rachis on the same frond, and there was no apparent trend towards localisation of CFDV-DNA in either leaf or rachis. Either tissue would, therefore, be suitable for assay. Both yellow and green tissue of symptomatic fronds was positive. Fronds 1 to 11 had a high frequency of detection when both leaf and rachis were considered. Trunk and secondary roots were the next most reliable (Table 3).

More detailed tissue sampling was done on two diseased PGD palms. Symptomatic fronds in position 9 were positive in leaflets at the tip, mid frond and base, and for rachis at mid

Table 3. *Frequency of detection of CFDV-DNA in tissue sampled from the infected coconut palms listed in Table 1*

Tissue	Leaf only[a]	Rachis only	Leaf + rachis	Palms positive[b]	Number tested[c]
Frond 1	6	6	7	19	27
3	5	4	8	17	27
5	5	5	10	20	27
7	6	3	10	19	26
9	6	5	10	21	27
11	3	4	8	15	20
13	3	4	6	13	21
15	2	3	5	10	18
17	3	1	3	7	14
19	2	1	3	6	10
21	0	0	3	3	4
23	0	1	2	3	4
25	0	0	2	2	5
27	0	0		0	1
Trunk				17	25
Primary root				13	21
Secondary root				18	28
Husk				6	17
Embryo				4	10

[a] Both leaf and rachis sampled from each frond, number of positives shown for each.
[b] Number of palms positive for the tissue tested.
[c] Number of palms tested for each tissue type.

frond and base. The sheath of the spathes in the axils of fronds 4, 8, 9 and 10 were all [illegible]. The stem of an opened inflorescence and small unfertilised and fertilised female buttons were negative. An unopened inflorescence was positive in the lower stem but negative at the tip which carried immature male florets.

Green husks were positive, whereas the meat of these nuts was negative. Polyethylene glycol 6000 (80 g/litre) precipitable material from the water of the nuts was also negative.

Table 4 shows an analysis of the distribution of CFDV-DNA in the fronds of a number of palms. The distribution pattern varied markedly both with and between fronds. Within leaflets, CFDV-DNA distribution was usually homogeneous. Both pre-mature (1 month before maturity) and mature nut bunches were assayed from 13 palms of different varieties. Husks of 64/65 of the nuts from the pre-mature bunches had CFDV-DNA. Husks of 43/53 nuts from the brown mature bunches were positive.

Assay of embryos and seedlings

Mature nuts were collected from 10 diseased palms. Four palms had positive embryos in the harvested nuts in the proportions 1/3, 1/6, 3/6 and 3/6. Of the remaining six palms, for which no positive embryos were detected, the numbers of embryos tested were 6, 5, 4, 3, 3 and 3. Out of a total of 45 embryos collected, eight were positive for CFDV-DNA. All of the positive embryos came from nuts with positive husks.

To determine whether seed transmission occurred, 19 seednuts were collected from several diseased trees and germinated. The husks of all nuts were positive for CFDV-DNA. Emerging leaves and rachis were negative for CFDV-DNA. This result agrees with an earlier trial in which 260 nuts were collected from diseased trees and another 260 nuts were collected from healthy trees and germinated. After 8 years, each group had the same number of diseased progeny (two), indicating that these cases arose from natural infection in the field (C. Calvez, J. F. Julia & J. P. Morin, unpublished result).

Assay of anthers and pollen

Pollen samples from five palms at the early stage of disease, comprising two CRD × NLD, two BGD and one RLT, were all negative for CFDV-DNA. Anther samples from three of these were positive.

In situ *localisation in coconut*

Autoradiography located CFDV-DNA over the vascular bundles of the rachis of a number of symptomatic fronds. Fig. 2 shows that the majority of bundles in a longitudinal section of rachis have a strong signal for CFDV-DNA. Not all bundles were positive, and the intensity of signal varied along the bundles. Transverse sections confirmed that not all bundles were positive. Leaf tissue from diseased palms which had been cut paradermally before hybridisation showed signals along some veins, and also isolated spots of high signal strength over the mesophyll.

Light microscopic examination of regions of sections positive for CFDV-DNA showed the accumulation of silver grains over phloem sieve tubes (Fig. 3). The signal was not detected over sclerenchyma, which was identified by its thick wall and staining with aqueous toluidine blue, nor over the xylem, which was identified by the presence of spiral and reticulate thickening.

Figs 4A and B show a lesion at the petiole base of frond 8 of a highly susceptible palm, and Fig. 4C shows a lesion on frond 11 of the same palm. Expansion of the lesion is associated with the collapse of fronds.

Table 4. *Distribution of CFDV-DNA within fronds of coconut palms*

Susceptibility to disease	CV.	Age at assay (Y)	Frond no.	Sampling Position on Frond: Base L[a]	Base R	Mid-base L	Mid-base R	Mid L	Mid R	Mid-Tip L	Mid-Tip R	Tip L	Tip R	Sampling position on leaflet from mid-frond: Base	Mid	Tip	Disease Status of Palm
High	MRD	4	4	1	1	1	1	1	1	1	1	1	1	1	1	1	Symptomatic
	MRD	4	4	0	0	0	0	1	0	1	0	0	0	1	1	1	"
	BGD	6	8	1	1	0	1	1	1	0	1	1	0	1	1	1	"
	TGD	4	4	1	1	1	1	1	0	1	0	1	0	1	1	0	"
	VTT × WAT	7	10	1	0	1	0	1	0	1	0	1	0	1	1	1	"
			16	1	0	1	0	1	0	1	0	1	0	1	1	1	"
	MRD	8	7	1	1	1	1	1	1	1	1	1	1	1	1	1	Remission
			26	0	0	0	0	0	0	0	0	0	0	0	0	0	"
	RLT	11	12	0	0	0	0	0	0	0	0	0	1	0	0	0	"
Low	BGD × VTT	5	4	1	1	1	1	1	1	1	1	1	1	1	1	1	Symptomatic
	BGD × VTT	4	4	1	1	1	1	1	1	1	1	1	1	1	1	1	"
	VRD	9[b]	9	0	0	0	0	0	0	0	0	0	0	0	0	0	Asymptomatic
			26	0	0	0	0	0	0	0	0	0	0	0	0	0	"

[a] Leaf (L) and rachis (R).
[b] This palm was positive in earlier samples from roots and fronds 1, 3 and 5 as shown in Table 1.

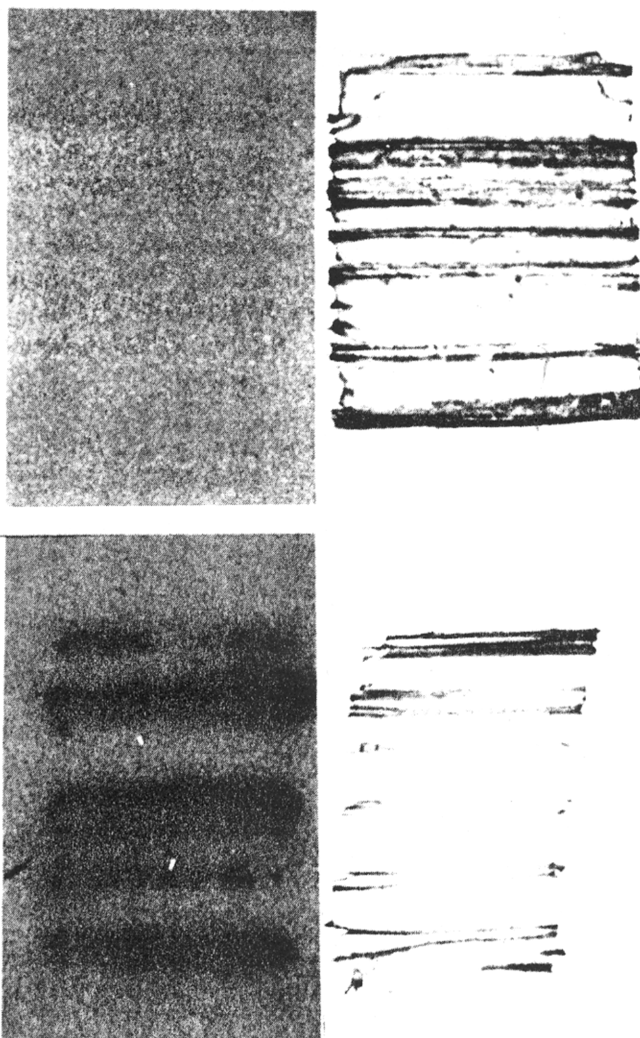

Fig. 2. Macro autoradiographs showing CFDV-DNA associated with the vascular bundles of the rachis of frond 6 of a healthy palm (upper), compared with that of a diseased PGD palm (lower). Corresponding autoradiographs and sections of rachis are shown at left and right, respectively.

Sections were cut from normal petiole and across the petiolar lesions to examine the relationship between signal strength and necrosis. As shown in Fig. 5, the necrotic zone on frond 8 showed a signal equivalent to that of non-necrotic tissue, whereas on frond 11, the necrotic region showed a very low signal in comparison with the non-necrotic tissue.

Localisation in Myndus taffini

The vector of CFDV retains inoculativity for 2 to 3 days (Julia, 1982), indicating that the mode of transmission is either semi-persistent or persistent. The *in situ* hybridisation technique was used to determine the proportion of *M. taffini* collected in the field which were positive for CFDV-DNA, and to attempt to localise it in the vector. Of 114 insect halves, 43 gave a signal for CFDV-DNA: of these, radioactivity was detected over the whole body of 28, over the abdomen of 14, and over the head of one.

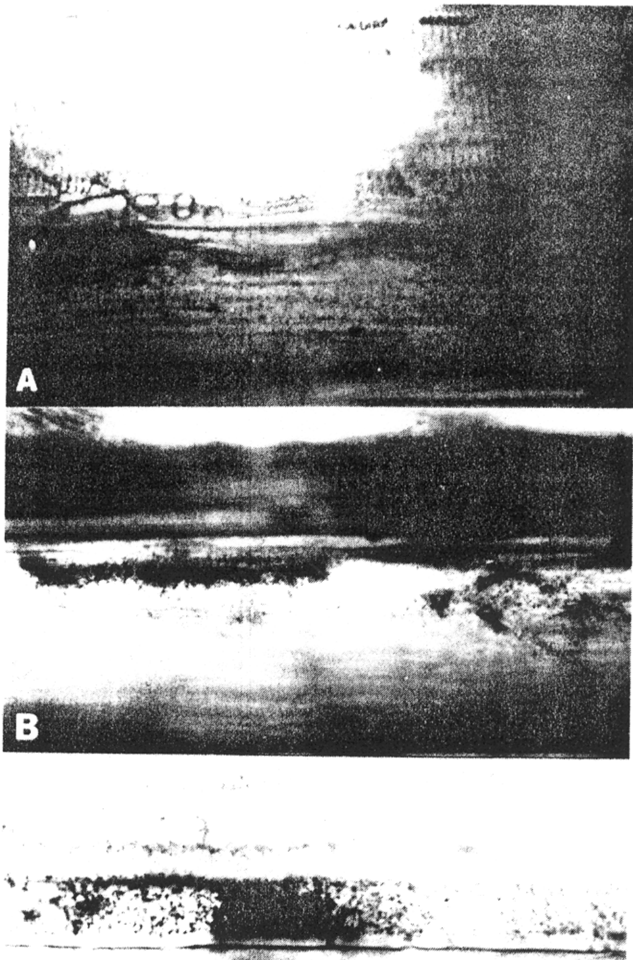

Fig. 3. Localisation of silver grains over the phloem in vascular bundles of the rachis shown in Fig. 2 by micro autoradiography. A. shows the uneven distribution of silver over the phloem (lower), with the xylem (upper) free of signal; B. shows signal over phloem adjacent to thick walled sclerenchyma (upper); C. shows a dense accumulation of silver over part of a phloem associated cell.

Discussion

Detection of CFDV-DNA by molecular hybridisation is the only assay available for CFDV. We have greatly improved this assay for CFDV (Hanold *et al.*, 1988) by using a 1203 bp DNA, amplified by PCR, from an encapsidated circular single-stranded 1291 nucleotide CFDV-DNA as a template for the synthesis of ^{32}P-labelled cDNA probes. The high specificity and sensitivity of the assay has allowed CFDV-DNA to be detected reliably, despite its low concentration in [illegible]

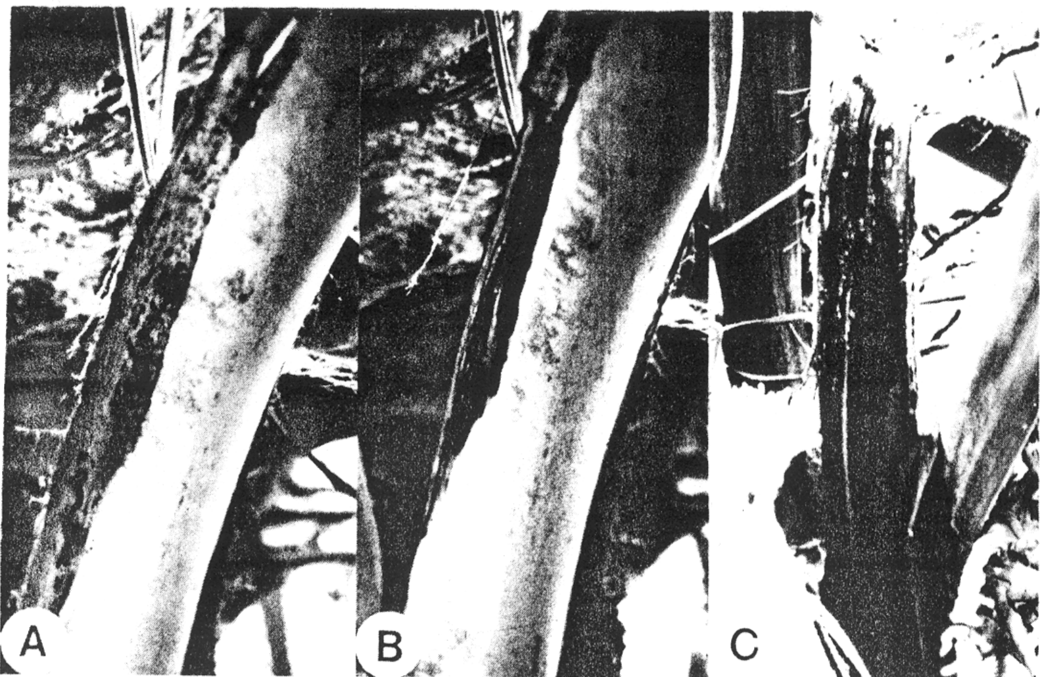

Fig. 4. A typical petiolar lesion associated with the yellowing and collapse of fronds in susceptible palms. A. MRD, at base of frond 8. B. As for A, with top layer of tissue removed to show internal necrosis. C. Frond 11 of the same palm showing collapse at the site of lesion.

for several applications reported in this paper:

- it has located CFDV-DNA in various tissues of palms and indicated the level of sampling needed for reliable diagnosis;
- it has shown the vascular location of CFDV in rachis and leaf;
- it has shown that CFDV-DNA can be detected in the body of the vector, and that in cases where the signal was sufficiently discrete it was over the abdomen rather than over the head.

In addition, a non-radioactive probing system has been developed as an alternative detection method.

Generally, all steps in diagnostic tests must be optimised. It was necessary to inhibit tannin production during crushing and to include an alkaline incubation step. Tests with the non-radioactive DIG-cRNA probe utilising antibody against the probe and an alkaline phosphatase based assay required a more rigorous alkali incubation than tests with the radioactive probe. This was necessary to prevent development of a non-specific endogenous reaction with the non-radioactive probing system. We have previously observed apparent endogenous phosphatase activity in coconut nucleic acid extracts (D. Hanold & J. W. Randles, unpublished), which was highly stable and interfered with colorimetric detection methods.

CFDV-DNA was distributed unevenly in whole plants and in an apparently non-systematic pattern. Thus, multiple sampling is needed for reliable diagnosis. The result of the examination of 29 whole palms indicated that root sampling should be combined with leaf sampling, particularly where new areas are being surveyed for CFDV incidence. We conclude that no preferred sampling position on leaflets or on fronds is indicated, but a minimum sampling should include tissue from fronds 1, 3 and 5, and secondary roots.

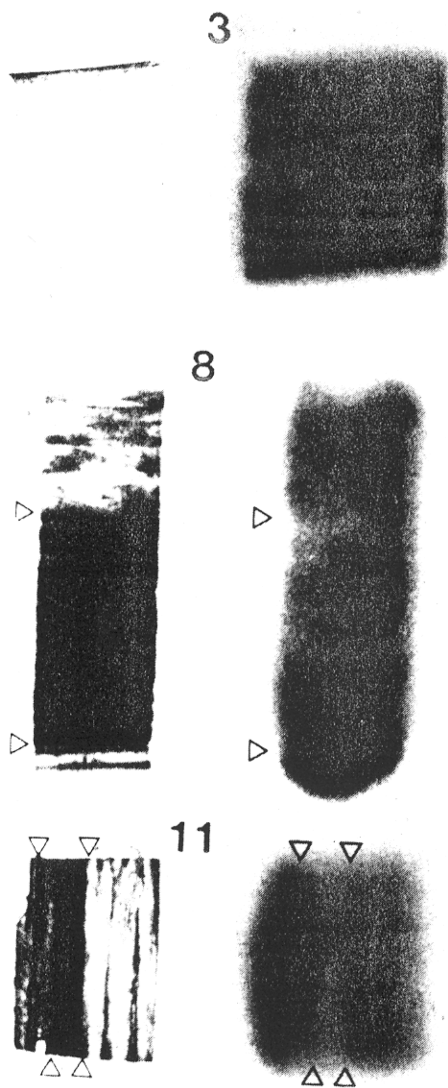

Fig. 5. Autoradiographs showing the distribution of CFDV-DNA in petioles of fronds 3, 8 and 11 of the palm in Fig. 4, in relation to the distribution of necrosis. Corresponding petiole sections and autoradiographs are shown on left and right, respectively. Arrows show the boundaries between dark necrotic and the non-necrotic tissue.

To localise virus-containing tissue, hybridisation and macro- and micro-autoradiography was done with unsupported intact sections of coconut rachis. This was possible because of the structural strength of this tissue. Mounting of tissue was necessary only for the application of gelatin and photographic emulsion. The location of the virus as indicated by the probe, in vascular bundles and phloem in particular, probably accounts for the low recovery of virus from tissue and the need to use alkali maceration to release CFDV-DNA for hybridisation assay.

The localisation of CFDV in phloem by electron microscopy alone would be very time consuming as indicated by the uneven distribution of probe between and along bundles.

Moreover, it is doubtful that the particles would be distinguishable from other cell components in thin sections. Autoradiography should assist in the location of virus-infected phloem for electron microscopic examination of virus infected cells. Higher resolution autoradiographic studies are needed as with abutilon mosaic geminivirus (Horns & Jeske, 1991), to identify intra-cellular sites of CFDV accumulation.

Symptoms of CFDV infection in highly susceptible cultivars are yellowing of leaflets in upper to central fronds, and progressive necrosis of the outer edge of the petiole (see Fig. 4). This necrosis is the site of collapse, causing the death of fronds. Autoradiography of a diseased palm showed that the virus DNA was present in a young unaffected frond, and both within and adjacent to the necrotic region at the early stages of lesion development (Fig. 5). It was not detected within the necrotic zone of an older frond of the same tree, but it was detected in the non-necrotic zones either side of this older lesion. These observations would be consistent with CFDV being associated with and causing this necrosis, then becoming degraded several months after the development of necrosis. No reason is known for the localisation of necrosis on petioles in a particular part of the crown. The only other symptom, namely, yellowing of leaflets, may be associated with cytopathic effects in phloem and reduced carbohydrate translocation as reported for the luteoviruses (Waterhouse, Gildow & Johnstone, 1988).

This study provides insight into some aspects relating to the poorly understood epidemiology of CFD. CFDV can be detected in leaves of seedlings within 6 months of controlled inoculation with *Myndus*, and symptoms appear 1–4 months later. Palms infected by natural exposure in the field show CFDV in most tissues sampled within 4–7 months (0.3–0.6 yr; Table 1) of symptoms first appearing. The disease-free trees, including tolerant cultivars, and palms in disease remission generally contain CFDV-DNA in leaflets and rachis, and a large reservoir of virus can, therefore, exist in apparently disease-free coconut growing areas.

Although CFDV DNA was detected in embryos, no transmission of CFD through seed has been demonstrated. No CFDV DNA was detectable in pollen samples. However, husks contain CFDV, and movement of nuts can allow transfer of virus to new sites. It will be necessary to determine whether vectors can acquire virus from green husk to decide whether there are quarantine risks from the movement of nuts.

The vector, *M. taffini*, commonly settles on coconut fronds at the junction of leaflet with rachis, where our *in situ* studies have shown that virus is likely to be available. Observations of feeding sites and behaviour should now be attempted to determine whether virus may be acquired by phloem feeding. The frequent detection of virus in coconut roots raises an alternative possibility that the soil-inhabiting larvae of *Myndus* could acquire virus from or infect, coconut roots during their pre-adult stage. We have not detected CFDV in the dicotyledon host of *Myndus* larvae, *Hibiscus tiliaceus*, but the frequently occurring proximity of coconuts to *Hibiscus* could allow acquisition of virus by larvae from roots of infected coconut palm growing adjacent to roots of *Hibiscus*. The association of CFDV-DNA with the abdominal areas of *Myndus* is consistent with accumulation of virus in the gut. Experiments now need to be done to determine whether the virus accumulates or replicates in the insect, and/or its eggs to determine whether it has a semi-persistent, circulative, or propagative association with its vector.

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