Detection and diagnosis of coconut foliar decay disease

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1. Introduction

Plant viruses are the second most important group of plant pathogens and may cause losses exceeding US$ 60 billion per year (Matthews, 1991). Their effects are most severe in agrarian economies where major staple crops are grown intensively. There are no direct control measures available for most crops, and resistance is available for only a few virus diseases where good crop research facilities are in place. Control is usually attempted through modification of cultural practices, but this requires an extensive knowledge of the disease cycle of the virus, including factors such as where the virus survives, the type of vector which transfers it from plant to plant, and the time and pattern of spread. A fundamental difficulty in working with viruses is that although their accurate identification is essential, the means of achieving this relies on laboratory based procedures. Symptoms are unreliable as either an indicator of the presence of a virus in a plant, or its identity.

The diagnosis of virus infection is done by detecting either the virus particles, or their components, such as their structural coat protein or their genome. Particles can be readily identified if they have a characteristic shape or size (Murphy *et al.*, 1995). Many viruses however occur at either very low concentrations in plant tissue, or their particles are not distinctive under the electron microscope. This difficulty is generally overcome by the use of specific antibodies if they are available to detect the coat protein of the virus particles in sap extracts, or the use of specific techniques for the detection of the unique genome of the virus in nucleic acid extracts of the plant tissue (Randles, 1993).

The use of nucleic acid based techniques led to the detection of a virus associated with coconut foliar decay disease (CFD) in Vanuatu (Randles *et al.*, 1986; Randles *et al.*, 1987; Randles and Hanold, 1989) and the sequencing of the virus associated DNA (Rohde *et al.*, 1990). The coconut foliar decay virus (CFDV) is the first virus of coconut palm to be characterised. The sequence information now available for CFDV provides access to a number of nucleic acid based diagnostic procedures. Some of these are described in this paper.

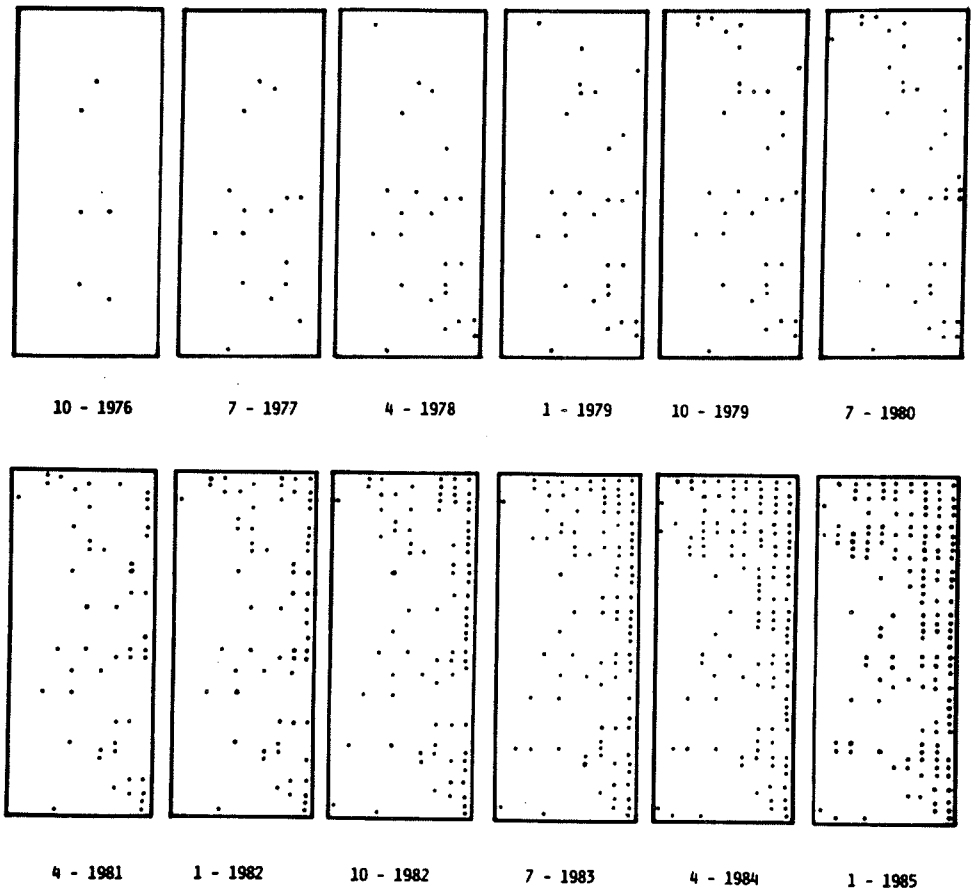

Figure 1. Progress of an epidemic of coconut foliar decay disease in a block of 400 Malayan Red Dwarf (MRD) coconut palms at the Vanuatu Agricultural Research and Training Centre between October 1976 and January 1985. Seedlings were planted in December 1974. The gradient developed from the boundaries closest to woodland including Hibiscus tiliaceus, whereas the lowest incidence was near boundaries adjacent to grassland. North is toward the bottom of the map. Results provided by courtesy of the Director, VARTC.

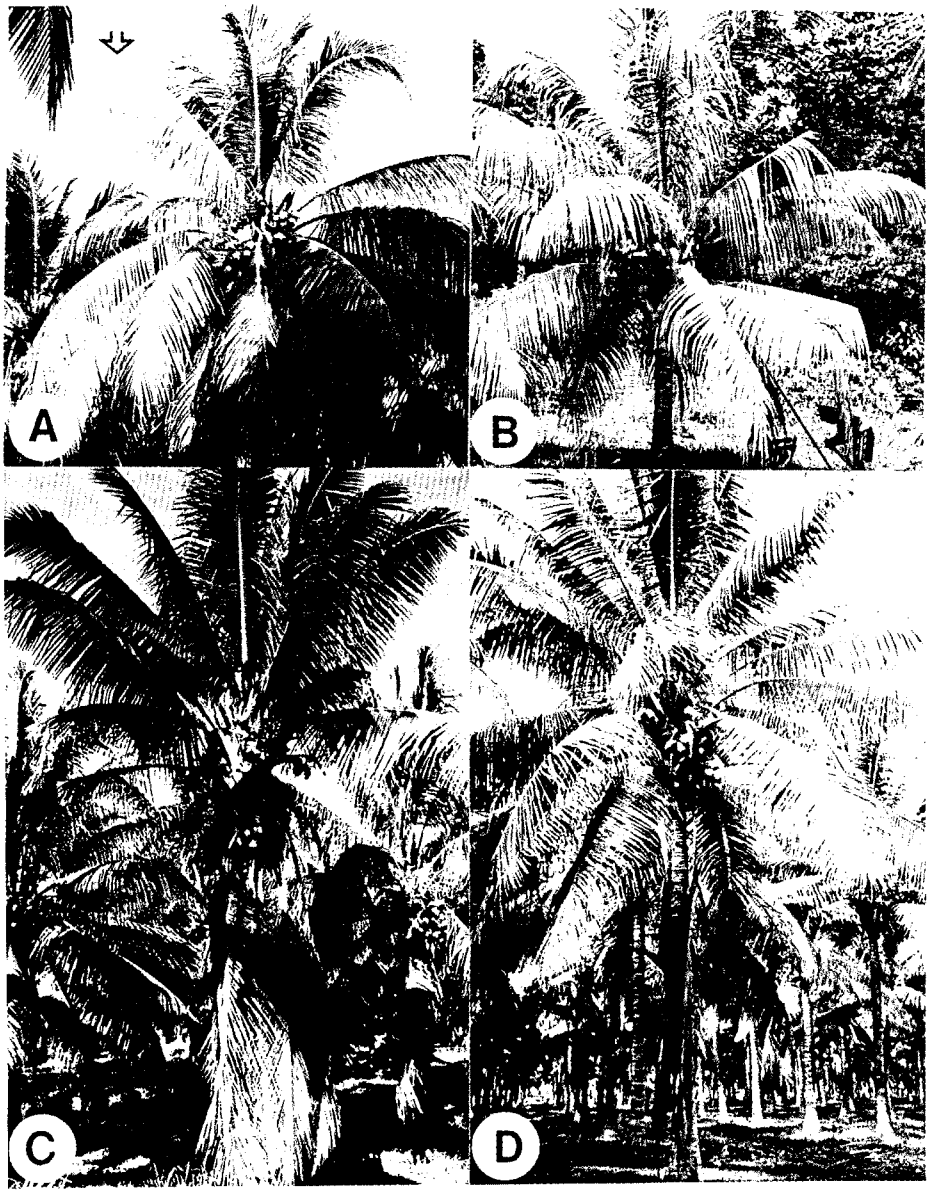

Figure 2. A comparison of symptoms of CFD on a range of naturally infected breeders lines at the VARTC. (A) MRD showing the initial yellowing of a frond (arrowed) near the centre of the crown. (B) MRD in remission from the disease. (C) A susceptible introduced tall variety showing typical collapse of fronds from the central whorl of the crown. (D) A disease resistant asymptomatic Vanuatu Tall palm.

2. Coconut foliar decay disease

CFD is a lethal disease of introduced coconut palm cultivars in Vanuatu. It was first reported in about 1964 in introduced Malayan germplasm. It spreads rapidly, as shown in Fig. 1 where the incidence reached 40% ten years after planting. The Malayan Red Dwarf variety is highly susceptible first showing early yellowing symptoms in the central crown (Fig. 2A) followed by a rapid decline and death of the tree (Fig. 3a). Affected palms sometimes show remission of symptoms (Fig. 2B). Other varieties are less severely affected (Fig. 2C) while the low-yielding local Vanuatu Tall (Fig. 2D) and Vanuatu Dwarf varieties remain disease-free (Calvez *et al.*, 1980) despite being infected with CFDV. CFD is economically important because it prevents the use of potentially more productive varieties for improvement of coconut palm by breeding. It is transmitted by the cixiid bug (plant hopper) *Myndus taffini* (Fig. 3b) (Julia, 1982; Julia *et al.*, 1985) which breeds on the roots of the tree *Hibiscus tiliaceus*, a common species in forest adjoining and within affected coconut plantations (Fig. 3a).

Symptoms are relatively distinctive as a first indication of disease. In the field, they first appear as yellowing of several leaflets in fronds 7 to 11 positions down in the crown from the unopened spear leaf (Fig. 2A). More general yellowing of the fronds ensues and they develop lateral necrosis of the petiole and die prematurely, hanging from the petiole downward through the canopy. Other fronds become yellow and die as they reach the central whorl of the crown, so that affected palms characteristically show a normal apex, several yellowish fronds, then several young dead fronds hanging through green older fronds. The trunk generally also narrows. Susceptible cultivars die 1 to 2 years after symptoms appear (Fig. 3a). Spathes at the base of yellow fronds rot if the subtending frond dies, or they produce an inflorescence that develops normally but which bears fewer nuts than normal.

3. Coconut foliar decay virus

A disease-specific single-stranded DNA of low electrophoretic mobility in polyacrylamide gels can be detected in palms with clear symptoms (Fig. 3d) (Randles *et al.*, 1986). It occurs in low concentrations only. Electron microscopy shows that these molecules are small and circular (Fig. 3e) (Randles *et al.*, 1987). Unusual 20-nm icosahedral particles that copurify with this DNA are considered to be the coconut foliar decay virus (CFDV) particles (Fig. 3c) (Randles and Hanold, 1989).

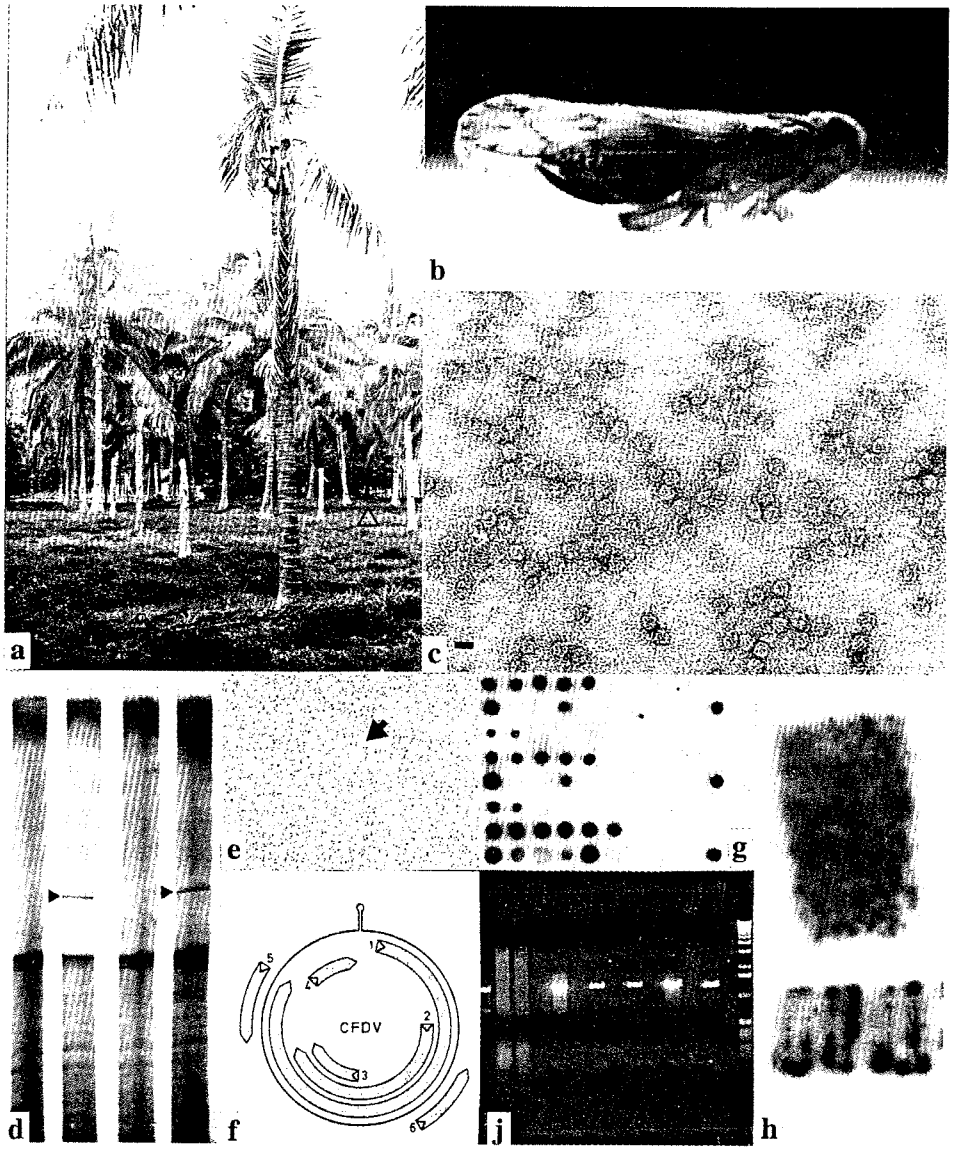

Figure 3. (a) Diseased palms in a commercial plantation. Note the *Hibiscus iliaceus* in the understory (arrowed). (b) Adult female *Myndus taffini*. (c) Negatively stained particles of coconut foliar decay virus. Bar represents 25 nm. (d) A denaturing polyacrylamide gel stained with silver, showing the single CFD associated DNA band in tracks 2 and 4 (arrowed). (e) Rotary shadowed circular molecule of CFDV DNA (arrowed). (f) Genome organisation of CFDV DNA showing the stem-loop structure near the presumed origin of replication, and the bidirectional arrangement of possible open reading frames. (g) Dot-blot assay of samples from infected and uninfected coconut palms, using a non-radioactive probe complementary to the sequence of CFDV DNA. (h) Distribution of CFDV DNA in transverse (upper) and longitudinal (lower) sections of rachis from a disease palm, showing the vascular location of the DNA. (i) Unilateral PCR of nucleic acid extracts from infected palms analysed on ethidium bromide stained agarose gels, showing the virus specific band midway down the gel.

The infectivity of the particles has yet to be tested, but their detection provides a possible way to develop a serological diagnostic test for the disease if sufficient amounts of virus can be prepared to immunise animals. CFDV particles are similar in size and symmetry to half-geminate (Hatta and Francki, 1979; Harrison, 1985) virus particles and to viruses such as subterranean clover stunt virus (SCSV) (Chu and Helms, 1988). CFDV differs from these other plant viruses in that the single-stranded circular DNA is intermediate in size between that of geminiviruses (ca 2,700 nucleotides) and SCSV (900-1,000 nucleotides). Moreover, SCSV and similar viruses have 6 or more circular single-stranded DNAs in the genome (Katul *et al.*, 1997), compared with the single DNA found in CFDV. CFDV appears to more closely resemble the porcine circovirus (Tischer *et al.*, 1982) in its genome organisation (Meehan *et al.*, 1997).

Purified CFDV DNA, amplified by the polymerase chain reaction, has been cloned, and the sequence determined by analysis of overlapping subgenomic cDNA clones (Hanold *et al.*, 1988; Rohde *et al.*, 1990). The complete sequence comprises 1291 nucleotides and contains open reading frames in both viral and antiviral sense for six potential proteins of molecular weight larger than 5 kDa (Fig. 3f). One of these (ORF1, 33.4 kDa) codes for a leucine-rich protein with the nucleotide triphosphate-binding motif GXGKS (glycine-x-glycine-lysine-serine) and may possibly participate in virus replication. The putative viral protein encoded by ORF3 (6.4 kDa) is a positively charged arginine-rich protein with homology to the capsid protein of nuclear polyhedrosis virus, and may represent the CFDV coat protein. CFDV DNA can form a stable stem structure of 10 GC base pairs subtending a loop sequence which in one orientation closely resembles the motif TAATATTAC conserved in a similar structural arrangement within the geminivirus group. The only additional significant sequence homology occurs in a stretch of 52 nucleotides which has 70% identity with a DNA containing badnavirus (Rohde *et al.*, 1995). Repeated stem-loop structures upstream from the 52 nucleotide sequence are consistent with the CFDV sequence having promoter activity.

4. Diagnosis of CFDV infection using DNA detection methods

4.1. Polyacrylamide gel electrophoresis (PAGE)

CFDV infection can be recognised in coconut palms by PAGE analysis of nucleic acids in the polyethylene glycol 6000 precipitable fraction of leaf, using silver staining. CFDV DNA migrates as a single band in denaturing gels (Fig. 3d), but

generally as two bands in non-denaturing gels (Randles *et al.*, 1987). Agarose gel electrophoretic methods are not sufficiently sensitive to detect the DNA. Routine diagnosis by PAGE does not prove that bands have nucleotide sequence similarity to CFDV. Therefore, hybridisation assays for CFDV DNA may need to be done in conjunction with PAGE.

4.2. Hybridisation with cDNA or cRNA

The high sensitivity and specificity of hybridisation assays is necessary to reliably detect CFDV DNA because of its variable and generally low concentration in coconut palm tissue. Two methods have been used for preparing probes for detecting CFDV by hybridisation. A PCR product representing 93% of the CFDV DNA sequence was synthesised and used as a template to produce a radioactive complementary DNA probe by transcribing with random primers, DNA polymerase and nucleotide triphosphates incorporating 32P. Alternatively, a digoxygenin (DIG) labelled complementary RNA probe was prepared by transcribing from a pSP65 vector containing the full sequence of CFDV DNA, with SP6 RNA polymerase and DIG-a UTP (Boehringer Mannheim) (Randles *et al.*, 1992). Probes detect CFDV DNA in Southern blots of fractionated nucleic acids from palm extracts, in dot blots of total nucleic acid extracts (Fig. 3g), and in thin sections of palm tissue (Fig.3h).

In order to prepare samples for the dot-blot procedure, extraction is done by crushing leaf or other tissue in one volume (w/v) of extraction buffer (50mM Tris-HCl, pH 7.2, 100mM Na acetate, 10mM EDTA, 0.1% monothioglycerol) in a plastic bag, treating an aliquot with 0.5N KOH for 2 hr at 25°C, extracting with phenol-chloroform, and precipitating with ethanol. The resuspended pellet is applied as 1 µl dots to a nylon membrane for the hybridisation step. The dots are fixed by baking at 80°C, prehybridised and hybridised with probe in the prehybridisation buffer under standard conditions (Randles *et al.*, 1992). Washing is done under stringent conditions of 0.018M Na+ at 65°C for 60 min before detecting the probe by either autoradiography, or the use of a colourimetric or light generating system for DIG-labelled probe. Alkali digestion at 37°C instead of 25°C has been found to be necessary when the non-radioactive system using alkaline phosphatase detection of DIG-cRNA is used, as endogenous enzyme activity in palm extracts is occasionally observed in the absence of this step. Southern blot analysis is done by fractionating the CFDV DNA containing nucleic acid extracts by either polyacrylamide or agarose gel electrophoresis, denaturing of the nucleic acids in 0.25-0.5N NaOH, capillary blotting or electrophoretic transfer to nylon membrane, and hybridisation (Hanold *et al.*, 1988).

For *in situ* studies, leaf or rachis samples are fixed in 1% glutaraldehyde, sectioned with a sledge microtome, treated with 0.25N KOH for 20-30 min at 22°C, then incubated in prehybridisation buffer for 5-24h at 42°C. Hybridisation is done with 32P labelled probe at 1x106 cpm / ml, and posthybridisation washes are done for an extended period to allow diffusion of unbound probe from the section. Sections can be either autoradiographed directly by covering with polythene film and exposure to X-ray film, or by attaching to microscope slides, coating sections first with gelatine gel to fill surface irregularities, then with liquid photographic emulsion. Exposure is in the dark at 4°C for at least 8 days, and the slides are developed and fixed before observation under the light microscope to detect silver grains. The location of the silver identifies the site of virus DNA accumulation (Randles *et al.*, 1992). A modification of this procedure has been used for localising the DNA in *Myndus* adults.

4.3. Use of PCR for diagnosis of CFDV

In recent years the polymerase chain reaction (PCR) has been increasingly used as a diagnostic tool for the detection of plant viruses. The sensitivity of PCR is several orders of magnitude higher than hybridisation diagnostic methods. For example 10 molecules can be amplified in less than 40 cycles to yield a product that is detectable in an ethidium bromide stained gel.

The successful amplification of a target sequence in PCR is dependent on numerous factors such as the type of thermal cycling equipment, the concentrations of reactants such as $MgCl_2$, dNTPs, the thermostable polymerase, the primers and template, and the structure and purity of the template.

The development of a PCR method for CFDV DNA has involved the testing of various methods for the extraction of total DNA. A satisfactory method is that described by Doyle and Doyle (1990) in that the cationic detergent CTAB (cetyltrimethylammonium bromide) solubilizes the plant membranes and forms a complex with the DNA. This method is fast and consistently gives a good yield of highly purified DNA from 1 to 10g of plant tissue. The fresh or frozen plant material (e.g. coconut palm leaves) is cut into small pieces and ground with mortar and pestle in the presence of liquid nitrogen. The ground tissue is incubated at 60°C in an appropriate amount of CTAB buffer [100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2 % (w/v) CTAB, 0.2 % (v/v) ß-mercaptoethanol (freshly added)] for 30 min with occasional stirring. The mixture is then filtered and extracted with an equal volume of chloroform/iso-amyl alcohol

(24:1). After precipitation of the DNA at room temperature by adding 0.8 volumes of propan-2-ol to the supernatant, the DNA is pelleted in a centrifugation step, washed once with 70 % ethanol, air dried, resuspended in TE/RNAse A (20 µg/ml) and incubated at 37°C for one hour. The RNAse is removed by phenol/chloroform/iso-amylalcohol (25:24:1) extraction. The DNA preparation is ready for use as a template in PCR after a final precipitation step.

Although a pair of oligonucleotide primers representing the putative stem-loop region of the known CFDV sequence can be used, the CFDV DNA molecule seems to have secondary structure that inhibits the complete extension of the complementary primer (even under high stringency conditions), with the result that the target sequence for the second primer is not produced. Therefore, we routinely use only the complementary primer to give linear (instead of geometric) amplification of CFDV DNA in a process that we term a unilateral PCR. A reaction volume of 40 µl is used. The reaction mixture comprises: 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM dNTP's, 10 pmol primer, about 5 ng of total DNA extract and 1 unit Tth thermostable DNA polymerase (Epicentre Technology, Madison, Wisconsin/USA).

The regime of amplification (after an initial denaturation step of 4 min at 95 °C) is 40 cycles of 20 s at 95 °C for denaturation, 20 s at 55°C for primer annealing and 90 s at 72 °C for primer extension. The reaction is completed by a final period of 15 min at 72 °C. The PCR products are analysed on 1 % agarose gels. The gels are stained with ethidium bromide and a single band of size ~ 1.3 kb is considered to be diagnostic for the presence of CFDV DNA in the extract (Fig. 3j). To confirm the identity of the predicted-size PCR-products as well as to detect further positive samples where no stained band is visible, the gels can be blotted onto nylon-membranes and hybridised with a radioactively labelled CFDV specific probe comprising a sequence other than the region containing the primer sequence.

4.4. A summary of results from the use of the diagnostic tests for CFDV

4.4.1. Dot blot assay

Using the hybridisation assay, CFDV DNA was found to be distributed unevenly in whole plants, 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. It was concluded that no preferred sampling position on

leaflets or on fronds could be suggested, but that a minimum sampling should include tissue from fronds 1, 3 and 5, and secondary roots (Randles *et al.*, 1992).

4.4.2. In situ localisation of CFDV

To localise virus-containing tissue, hybridisation and macro- and micro-autoradiography can be done with unsupported intact sections of coconut rachis. This is possible because of the structural strength of this tissue. Mounting of tissue is necessary only for the application of gelatin and photographic emulsion. The virus was located in vascular bundles and phloem in particular, probably accounting for the low recovery of virus from tissue and the need to use alkali maceration to release CFDV DNA for hybridisation assay (Randles *et al.*, 1992).

Autoradiography of tissue from a diseased palm has shown that the virus DNA is present in young symptomless fronds, and both within and adjacent to the necrotic region of the petiole at the early stages of lesion development. It has not been 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 explanation is available for the localisation of necrosis on petioles in a particular part of the crown. The only other symptom, yellowing of leaflets, may be associated with cytopathic effects in phloem and reduced carbohydrate translocation.

4.4.3. Detection of CFDV in asymptomatic palms

CFDV can be detected in leaves of seedlings within 6 months of controlled inoculation with *Myndus*, and symptoms appear 1-4 months later. Palms infected by natural exposure in the field show CFDV in most tissues sampled within 4-7 months of symptoms first appearing. CFDV-DNA has been found in leaflets and rachis of disease-free trees. These include tolerant cultivars in areas of disease incidence and palms in remission from CFD. Therefore, a large reservoir of virus can exist in apparently disease-free coconut growing areas.

4.4.4. CFDV in embryos

Although CFDV DNA can be detected in embryos, no transmission of the disease through seed has been demonstrated. No CFDV DNA has been detected in pollen

samples. However, husks contain CFDV DNA, and movement of nuts may therefore allow transfer of the virus to new sites. It may be necessary to determine whether vectors can acquire virus from green husk when evaluating quarantine risks from the movement of nuts.

4.4.5. CFDV in the vector

The vector, *M. taffini*, commonly settles on coconut fronds at the junction of leaflet with rachis, where in situ studies have shown that virus is likely to be available. *Myndus* can also be reared artificially (Morin, 1993). Hybridisation assays indicate that CFDV-DNA is associated with the abdominal areas of *Myndus* adults collected in the field, and in the gut (Randles and Miller, unpublished results). Experiments using PCR have detected virus in adult males and females, and in nymphs (E.Wefels, unpublished results). Further experiments need to be done to determine whether the virus accumulates or replicates in the insect, and/or its eggs. This would allow the mode of transmission to be determined, that is, whether the virus has a semi-persistent, circulative, or propagative association with its vector.

4.4.6. Natural host range

The host range of CFDV remains to be determined by PCR assay to identify the virus reservoir species.

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