Association of Single-Stranded DNA with the Foliar Decay Disease of Coconut Palm in Vanuatu

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ABSTRACT

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Foliar decay disease induced by feeding of the cixiid plant hopper, *Myndus taffini*, is a disease of unknown etiology that develops in coconut palms in Vanuatu. Extraction of diseased leaf tissue with or without a cellulase digestion treatment, followed by a precipitation step with 8% polyethylene glycol 6000, and high-speed centrifugation, yielded a sedimentable component containing a single-stranded DNA. This component has an estimated sedimentation coefficient of less than 75 S, and buoyant densities in Nycodenz and Cs_2SO_4 of 1.21–1.25 g/ml and about 1.36 g/ml, respectively, but no virus-like particles were specifically associated with this component. An assay for the DNA, based on polyacrylamide gel electrophoresis and silver staining, showed that it was disease specific. It has an estimated mol wt between 2 and 3×10^6 in 3.3% polyacrylamide gels and therefore appears not to be typical of DNA from any plant virus taxonomic group. The use of this DNA for the diagnosis of foliar decay disease and the possibility that it indicates a virus etiology is discussed.

Foliar decay is a disease of introduced coconut palm cultivars in Vanuatu that is induced by feeding of the cixiid bug (plant hopper), *Myndus taffini* Bonfils (2,7,8). It is also known as New Hebrides coconut disease (2) and foliar decay of coconut caused by *M. taffini* (8). The disease is economically important because of its present influence on a regional coconut palm selection and breeding program in Vanuatu.

A range of symptom severities is observed in affected introduced cultivars and their hybrids, whereas the local *Cocos nucifera* L. 'Vanuatu Tall' is symptomless and its hybrids show mild symptoms (1,8). The cultivar Malayan Red Dwarf (MRD) is always highly susceptible to foliar decay disease (FDD) in the field (Fig. 1) and is used as an indicator plant for epidemiological and insect transmission studies. In the field, yellowing first appears in several leaflets of the fronds seven to 11 positions down in the crown from the unopened spear leaf. More general yellowing of the fronds ensues and they develop lateral necrosis of the petiole and die prematurely, hanging from the petiole downward through the canopy. Other fronds become yellow and die as they reach position seven to 11 in 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 narrows and may thicken again if remission occurs, as in a tolerant palm. Susceptible cultivars die between 1 and 2 yr after symptoms appear. Spathes at the base of yellow fronds rot if the subtending frond dies, or they produce an inflorescence that develops normally but that bears fewer nuts than normal.

Seedlings that are experimentally inoculated in insect-proof cages with *M. taffini* collected in the field show similar symptoms on fronds three to five from 6 to 11 mo after inoculation (Figs. 2 and 3). Collapse of the yellow fronds seems to coincide with some lateral necrosis at the base of the petiole.

Neither fungi, bacteria, nor nematodes have been implicated as the pathogen, and tetracycline applications at rates used to control coconut lethal yellowing had no effect on disease progress (8).

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Fig. 1. Symptoms typical of a foliar decay outbreak in the field. The first four palms in the row are the highly susceptible Malayan Red Dwarf cultivar, whereas those behind are the resistant Vanuatu Tall. The first palm shows the small crown and hanging older dead fronds typical of the disease. The dead palms were probably infected earlier. *Hibiscus tiliaceus* (burao) forms the bushy fence line in the background and is a host of the vector of foliar decay disease, *Myndus taffini*.

Electron microscopy has failed to detect any microorganism or virus in affected tissue, and attempts to detect the coconut cadang-cadang viroid by either gel electrophoretic analysis or molecular hybridization assay of nucleic acid extracts (11,13; Randles and Dollet, *unpublished*), have been negative.

The correlation between the distribution of diseased palms and *M. taffini* in plantations and the demonstration of efficient transmission of FDD by adults of *M. taffini* (8) suggests that the disease is caused by a transmissible agent. It seems unlikely that an insect toxin is involved because small groups of *M. taffini* can transmit FDD during a short inoculation feed (8).

This paper describes attempts to implicate a pathogen by analyzing palm extracts for disease-specific components. We report the association of an unusual single-stranded DNA component with inoculated seedlings and discuss the possibility that FDD has a viral etiology.

MATERIALS AND METHODS

Source of infected material. MRD seedlings at the two- to three-leaf stage were inoculated by caging *M. taffini* adults collected from the field on the young shoot (8). MRD seedlings infected naturally by exposure in the field were also used. Yellowing leaf tissue was collected 6–8 mo later from fronds two, three, or four of diseased palm. Control tissue was collected from uninoculated seedlings grown in the same nursery at Saraoutou, Espiritu Santo, Vanuatu (formerly New Hebrides), or from healthy seedlings grown in the glasshouse at the Waite Institute.

Fig. 2. Malayan Red Dwarf seedling infected in an insect-proof screenhouse by *Myndus taffini* collected on diseased coconut palms in the field. Frond 3 (arrowed) shows yellowing with light brown patches.

Midribs were removed, leaflets were air freighted to Adelaide, surface sterilized with sodium hypochlorite (1% available chlorine), ethanol, and distilled water in the ratio 1:2:2, rinsed, drained, and either extracted immediately or stored at −20 C.

Extraction procedures. Leaflets were cut with scissors and immediately blended in a blender with buffer in the ratio 20 g to 120 ml of buffer. Several alternative methods were used.

Method A. The blending buffer was 0.1 M sodium citrate, pH 4.7, containing 10 mM EDTA, 1% monothioglycerol (Sigma Chemical Co., St. Louis, MO) and 0.5% cellulase (Sigma). The extract was incubated at 30 C for 4 hr, the pH was adjusted to 7.0 by adding 0.5 M Na_2HPO_4, and the incubation was continued at 4 C for 16 hr. The extract was blended again, strained through muslin and polyethylene glycol 6000 (PEG; British Drug House, average mol wt 6,000–7,500) was added to 8%. The resulting precipitate was collected by centrifugation at 10,000 *g* for 10 min, resuspended in 20 ml of 10 mM phosphate buffer, pH 7.6, for 2 hr, clarified at 10,000 *g* for 10 min, and centrifuged at 200,000 *g* for 70 min. Pellets were resuspended in buffer, clarified as above, and centrifuged at 200,000 *g* for 90 min through a 20% sucrose cushion. The final pellet was resuspended in 0.1 ml of 10 mM phosphate buffer and clarified as above, and nucleic acid was extracted from the supernatant phase by incubation with 0.5 ml of 0.1% protease, from *Streptomyces griseus*, 0.1 M sodium acetate and 0.5% sodium dodecyl sulfate (SDS) for 16 hr at 37 C (11). This was extracted

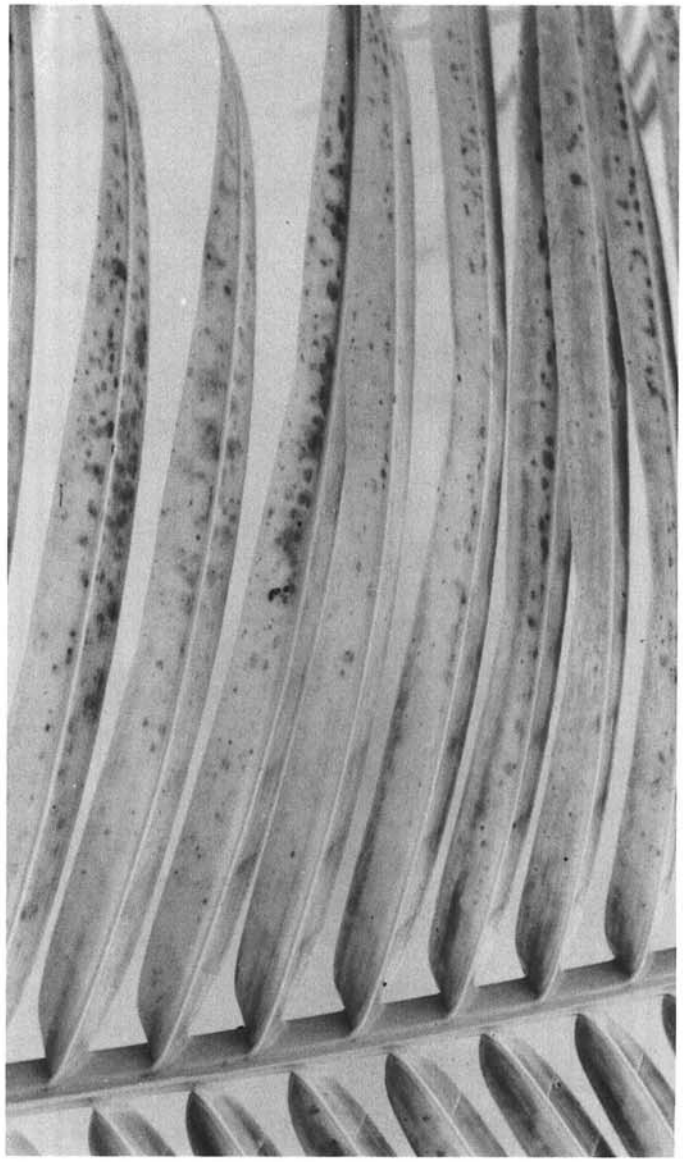

Fig. 3. Symptoms on frond 3 of the seedling in Figure 2.

with 0.5 ml of an aqueous phenol-cresol mixture (9:1), and nucleic acids were precipitated from the supernatant with three volumes of ethanol. Pellets were resuspended in 50% glycerol containing xylene cyanole and electrophoresis buffer.

Method B. Leaf tissue was blended in 0.1 M phosphate buffer, pH 7.0, containing 10 mM EDTA and 1% monothioglycerol, the extract was strained through muslin, and the filtrate was stirred in the presence of 2% Triton X-100 for 1 hr at 4 C. It was clarified by centrifugation at 10,000 *g* for 10 min, PEG was added to 8%, and the precipitate was collected by low-speed centrifugation as above. The pellet was resuspended in 10 mM phosphate buffer, pH 7.6, for 2 hr, clarified by low-speed centrifugation, and centrifuged at 200,000 *g* for 70 min. The pellet was resuspended in 1 ml of 2% SDS, mixed with an equal volume of 90% phenol, and shaken for 45 min at 20 C. After centrifugation, the aqueous phase was reextracted with an equal volume of a 1:1 phenol-chloroform mixture, and nucleic acids were collected by ethanol precipitation.

Method C. Leaf was extracted as for method B but was clarified by mixing with 2% Triton X-100 for 15 min at 4 C followed by the addition of 2 g of bentonite (Sigma) and mixing for another 2 min. The mixture was centrifuged at 10,000 *g* for 10 min, PEG was added to the supernatant to a concentration of 8%, and the

TABLE 1. Association of high molecular weight DNA with foliar decay disease

Disease status	Site	Extraction method[a]	No. of palms Assayed	No. of palms With DNA
Asymptomatic	Waite Institute	A	5	0
	Vanuatu	A	4	0
	Vanuatu	B	3	0
	Vanuatu	C	2	0
	Vanuatu	D	2	0
Total			16	0
Symptomatic Inoculated	Vanuatu	A	29	28
	Vanuatu	B	12	12
	Vanuatu	C	10	10
Uninoculated	Vanuatu	B	1	1
	Vanuatu	D	2	2
Total			54	53

[a]See Materials and Methods.

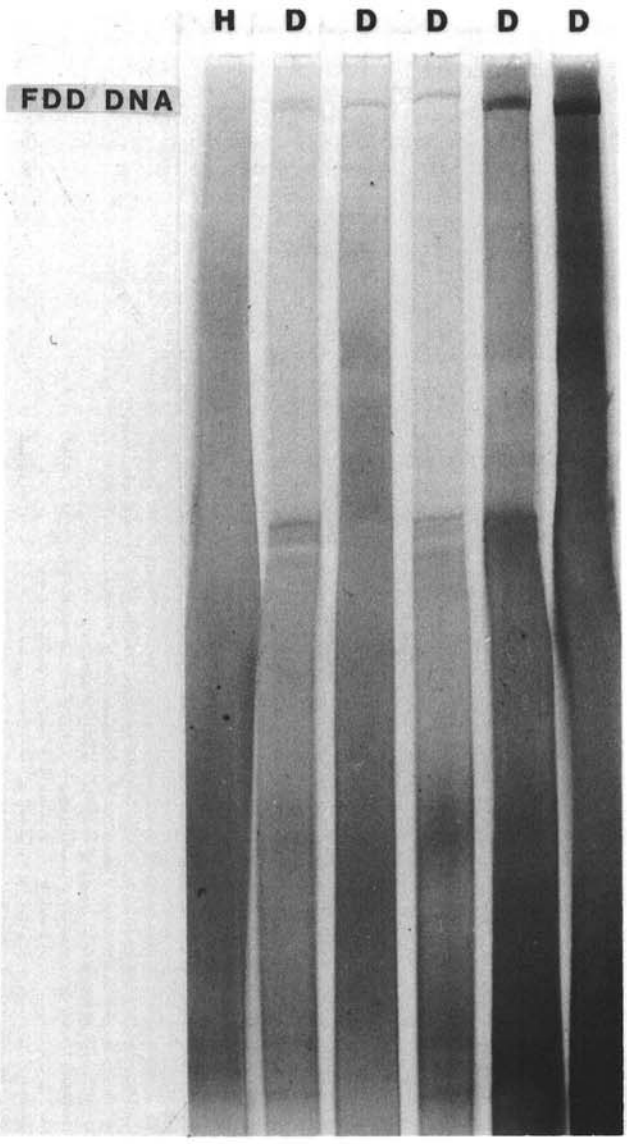

Fig. 4. Specific association of a single band (FDD-DNA) with nucleic acid extracts from diseased (D) but not healthy (H) coconut seedlings. Electrophoresis was in a 5% polyacrylamide gel containing 8 M urea.

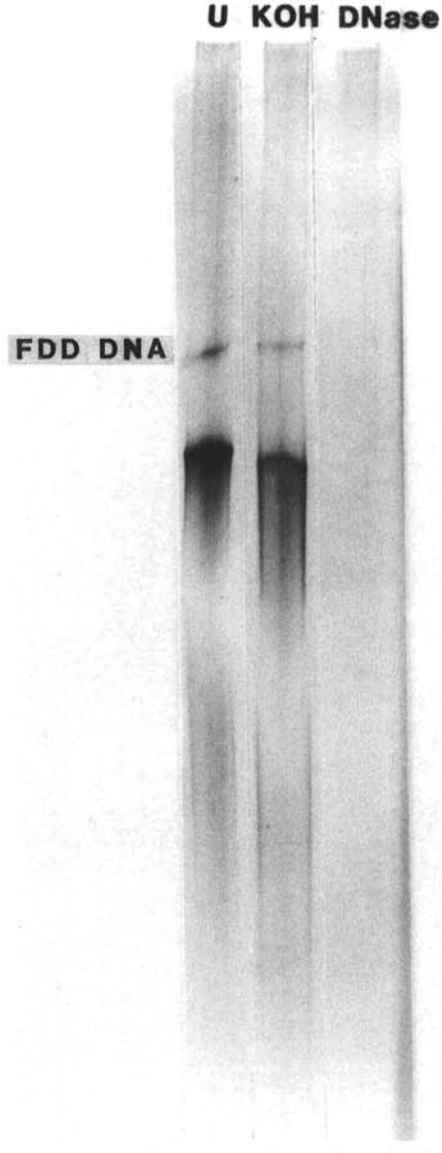

Fig. 5. Alkali resistance (KOH) and deoxyribonuclease (DNase) sensitivity of FDD-DNA compared with an untreated sample (U). Electrophoresis was in a 5% polyacrylamide gel containing 8 M urea.

precipitate was collected after 90 min by low-speed centrifugation. Nucleic acids were extracted as with method B.

Method D. Extractions were done as with method C, except that the first PEG precipitate was resuspended in 20 ml of 10 mM phosphate buffer, pH 7.6, by shaking for 1–2 hr, and clarified by low-speed centrifugation. A second 8% PEG precipitate was obtained from the supernatant as before, and this pellet was resuspended in the minimum volume of sterile 10 mM phosphate buffer, pH 7.6. An aliquot of this was mixed with an equal volume of a mixture of protease (2 mg/ml), 0.5% SDS, 0.1 M sodium acetate, and 10% glycerol, incubated at 37 C for 1–2 hr, or at 25 C for 16 hr, mixed with a trace of marker dye and subjected directly to polyacrylamide gel electrophoresis.

Centrifugal methods. Rate zonal density gradients were linear 10–40% sucrose in 10 mM phosphate buffer, pH 7.6, centrifuged in a SW 65 Spinco rotor at 60,000 rpm for 2 hr at 5 C. Sedimentation rate markers were velvet tobacco mottle virus (115 S) (12), and fraction I protein (18 S) and 80 S ribosomes prepared by extracting young leaf of *Nicotiana clevelandii* Gray in one volume (w/v) of 10 mM phosphate buffer. Gradients were fractionated with an ISCO apparatus.

Isopycnic density gradients of Nycodenz (4) were set up by layering equal volumes of 30, 40, 50, and 60% (w/v) Nycodenz in phosphate buffer in 5-ml ultraclear (Beckman Instruments, Inc., Palo Alto, CA) centrifuge tubes. Samples of 100–400 μl were overlaid, and centrifuged at 35,000 rpm for 16 hr at 5 C in a SW 50.1 Spinco rotor followed by slow deceleration. Bands observed by light scattering were recovered drop-wise from a bottom puncture or by aspiration from the top with a glass pipette or syringe and needle. Cs_2SO_4 gradients were prepared by layering 1.7 ml each of 50% and 30% Cs_2SO_4 in 10 mM phosphate buffer, applying 0.2–0.3 ml of sample, and centrifuging as above.

Polyacrylamide gel electrophoresis. Slab gels, 1.5 mm thick and 10 cm long, containing either 3.3 or 5% polyacrylamide and 8 M urea, and buffered at pH 8.3 with 90 mM tris, 90 mM boric acid, 3 mM EDTA were used in a Bio-Rad 220 apparatus to fractionate nucleic acids. Marker DNAs were Hind III restricted fragments of λ phage DNA (New England Biolabs) or Eco R1 fragments of SPP1-DNA (from A. Rezaian) heated at 100 C for 3 min with or without added 50% deionized formamide. Five-percent gels were run at 18 mA constant current for 16 hr; 3.3% gels were run at 30 mA for 3 hr. Silver staining was a modification of the method of Sammons et al (14) with a 1-hr wash in two changes of 10% ethanol–0.5% acetic acid, a 1-hr stain in 0.2% $AgNO_3$, two brief washes in degassed distilled water, and development in a solution of 0.375 N NaOH, 2 mM sodium borohydride, and 0.15% formaldehyde. Gels were left in 70 mM Na_2CO_3 for 1 hr, and stored in 1% acetic acid.

Identification of nucleic acids. Sensitivity to alkali was tested by incubation of nucleic acid preparations with 0.3 N KOH at 37 C for 1 hr, neutralization with 1 N HCl and precipitation with ethanol before analysis.

Sensitivity to ribonuclease A was tested by incubation with boiled RNase A followed by addition of phenol to 18% to stop the reaction. Sensitivity to deoxyribonuclease I was tested by incubation with 100 μg of the enzyme DNEP (Sigma) per milliliter of 100 mM Tris-Cl, pH 7.4, and 100 mM $MgCl_2$, with termination as for ribonuclease. Alternatively, 20 μg of enzyme per milliliter of 10 mM Tris-Cl and 10 mM $MgCl_2$ was used at 37 C for 60 min before ethanol precipitation of residual nucleic acid. Single-strandedness was tested by incubating preparations with S_1 nuclease (Sigma) at 100 units/ml with buffer containing 30 mM sodium acetate buffer, pH 4.6, 3 mM $ZnSO_4$, 40 μg denatured calf thymus DNA per milliliter, and either 50 mM or 300 mM NaCl; incubation was at 37 C for 10 min. Double-stranded DNA was melted at 100 C for 5 min and was used as a test of enzyme activity.

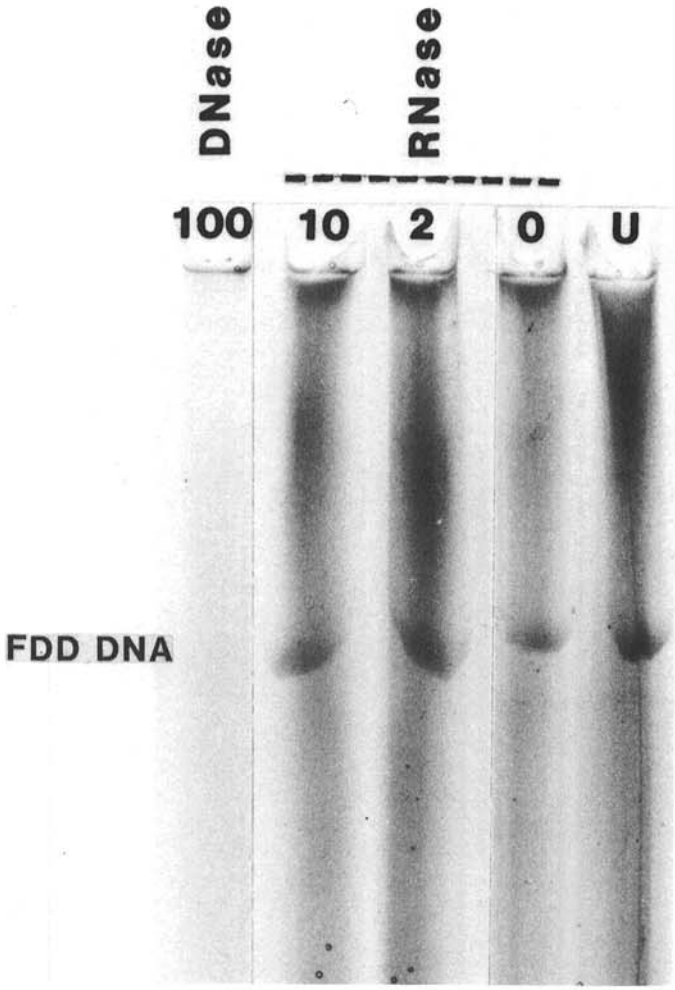

Fig. 6. Insensitivity of FDD-DNA to ribonuclease A (RNase) at 2 and 10 μg/ml, incubated at 20 C for 40 min, compared with an untreated sample (U) and a sample incubated without enzyme (O). The band is identified by its sensitivity to DNase I (100 μg/ml). Electrophoresis was in a 5% polyacrylamide gel containing 8 M urea.

RESULTS

Detection of disease-associated DNA. Method A was similar to that adopted for purifying a luteovirus (6) and was used to ensure complete extraction of tissue; methods B, C, and D were much shorter, and C and D could be completed within a day.

All four extraction methods yielded a disease-associated high molecular weight nucleic acid band (FDD-DNA; Table 1, Fig. 4). The high molecular weight FDD-DNA band was present at a very low concentration because it was only detectable by silver staining, not with ethidium bromide or with toluidine blue O. It was readily identified because it was the uppermost band under the conditions of electrophoresis used.

Several variations in the extraction method were tested. Dark background staining of gels was greatly reduced by the incorporation of bentonite in the extract at the clarification step and helped detection of FDD-DNA. Thioglycerol and 1% 2-mercaptoethanol could be interchanged in the extraction buffer to prevent browning of the coconut leaf extract; but even when reducing agents were omitted, thus leading to browning of extracts, FDD-DNA was detectable when bentonite clarification was used. Activated charcoal (5%), DEAE-cellulose powder (1%) and celite powder (10%) also provided some clarification when mixed with and removed from the extracts by centrifugation (3) but seemed to be less effective than bentonite. The organic solvents, 1-butanol (8.5% for 10 min at 4 C) and chloroform (0.5 volumes, emulsified for 5 min at 4 C) gave lower yields than Triton X-100 (2%), without improving clarification. Phenol, protease, and alkali (0.3 N NaOH, 37 C for 2 hr, neutralization with HCl) treatments were all effective for extracting FDD-DNA from final pellets.

The band was recovered from extracts of fresh or frozen leaf tissue. Moreover, FDD-DNA was isolated from samples weighing

2, 5, 10, or 20 g when blended in 120 ml of buffer, using method D. The DNA was also isolated from samples stored at 4 C for 4, 9, and 12 days before extraction with method B; but background staining with silver in the gel was heavier for the 9- and 12-day samples, interfering with the detection of the DNA.

Properties of FDD-DNA. The insensitivity of the FDD-DNA band to RNase A and alkali, and its sensitivity to DNase I (Figs. 5 and 6), confirms its identity as DNA.

Heating the preparations at 100 C with or without added 50% formamide had no effect on the electrophoretic mobility of FDD-DNA (Fig. 7) consistent with it being single-stranded. Marker DNA fragments from SPP1 and λ bacteriophages were melted to single-stranded DNA by these treatments and migrated more slowly than the double-stranded forms. Single-strandedness of the FDD-DNA was confirmed by treatment with S_1 nuclease. The enzyme removed FDD-DNA after incubation in both 50 mM and 300 mM NaCl. Restriction fragments of double-stranded λ phage DNA were unaffected under the conditions used, whereas single-stranded fragments produced by heating the marker to 100 C for 5 min were sensitive to the enzyme.

FDD-DNA migrated more slowly than the 23.13 Kb fragment of λ phage DNA in 5% gels, but in 3.3% gels, it migrated at the rate expected for a λ fragment between 6.56 and 9.42 Kb in length. Double or triple bands were sometimes seen at the FDD-DNA position in 3.3% gels. A value of 8.7 Kb was estimated from two trials (Figs. 7 and 8), but the dependence of apparent molecular weight on gel concentration requires further investigation.

Sedimentation rate and density of the FDD-DNA containing component. Extracts prepared by method B to the step before deproteinization were analyzed by rate zonal and isopycnic density gradient centrifugation.

In several experiments, FDD-DNA was recovered from sucrose density gradients in the zone representing sedimentation coefficients between 16 and 75 S. The lowest values are close to those expected for DNA alone, whereas the highest could represent a particulate component containing FDD-DNA. Material collected by sedimentation from the 30–80 S region of a sucrose density gradient had a buoyant density in Nycodenz within the 1.22–1.27 g/ml range, as determined by gel electrophoretic analysis of fractions for FDD-DNA. The FDD-DNA containing component had the same density range when preparations were analyzed directly on Nycodenz gradients (Fig. 9). In Cs_2SO_4 gradients, FDD-DNA was recovered from the region of density about 1.36 g/ml.

Spherical particles about 20 nm in diameter were sometimes observed in these fractions. However, gel electrophoresis analysis of proteins from sucrose and Nycodenz fractions from healthy and diseased palms showed that both had numerous bands, and no

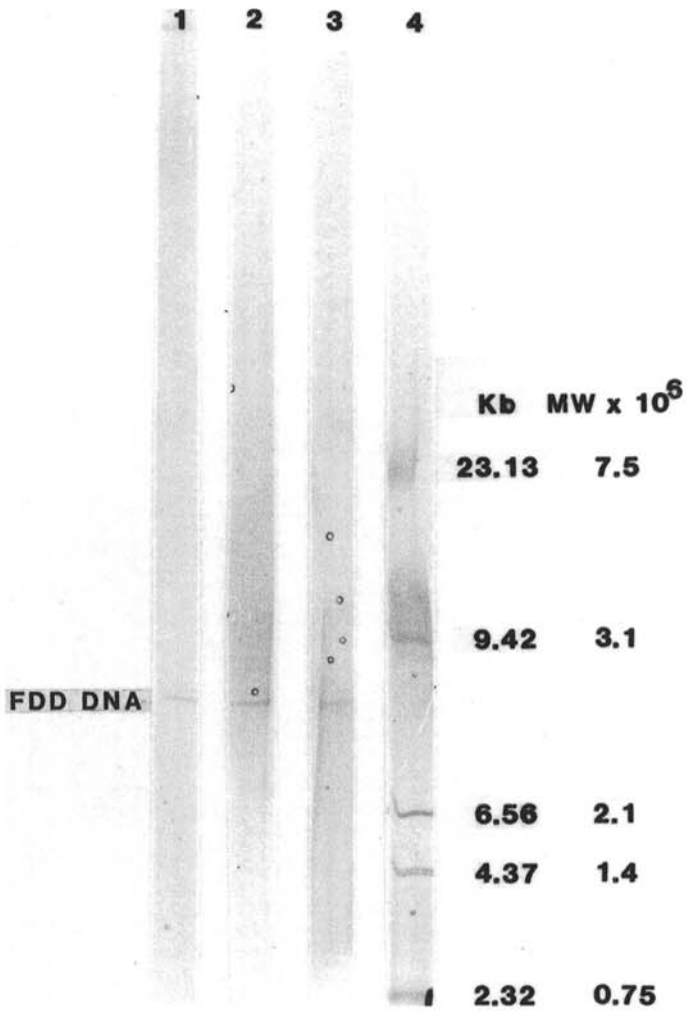

Fig. 7. Electrophoretic mobility of untreated (lane 1), heated (100 C, 5 min; lane 2), and fully denatured (in 50% formamide, 100 C, 5 min; lane 3) foliar decay disease-DNA, compared with that of single-stranded marker DNA prepared by boiling Hind III restriction fragments of phage λ DNA. Length (Kb) and molecular weight of marker DNA was checked by coelectrophoresis with single-stranded fragments of an Eco R1 digest of phage SPP1 DNA. Analysis was in a 3.3% polyacrylamide gel containing 8 M urea.

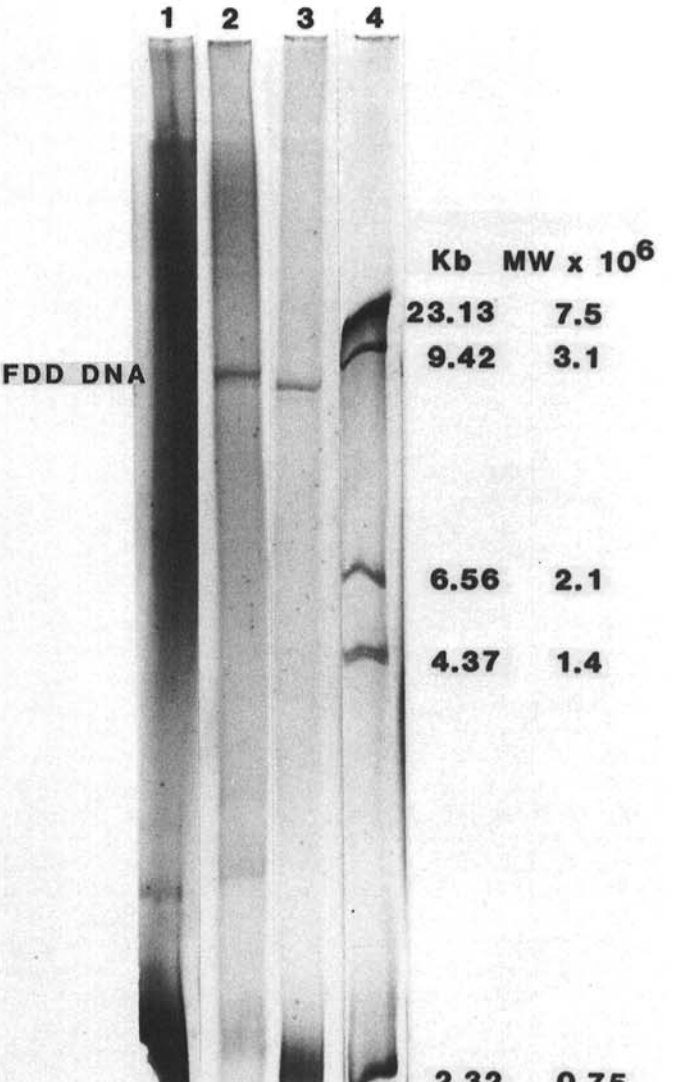

Fig. 8. Analysis of an extract (method B) from a healthy palm (lane 1), foliar decay disease (FDD)-DNA from the 1.26-g/ml density fraction of a Nycodenz isopycnic gradient (lane 2), and FDD-DNA treated with RNase at 2 μg/ml for 15 min at 37 C (lane 3). Length (Kb) and molecular weight of marker DNA was checked by coelectrophoresis with single-stranded fragments of an Eco R1 digest of phage SPP1 DNA (lane 4). Analysis was in a 3.3% polyacrylamide gel containing 8 M urea.

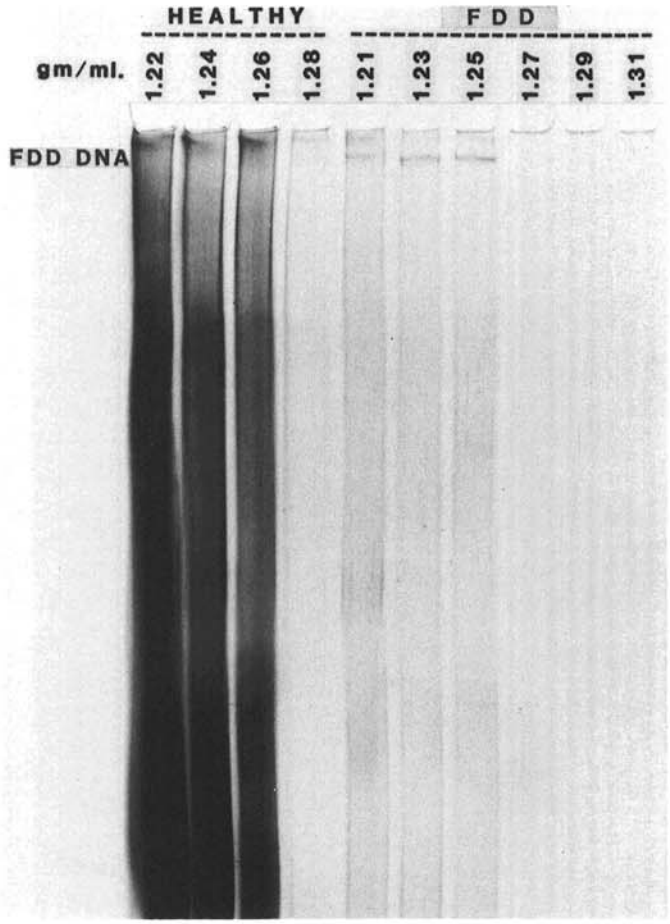

Fig. 9. Distribution of the foliar decay disease (FDD)-DNA containing component in a Nycodenz isopycnic density gradient. A diseased (FDD) and a healthy palm were extracted by method B to the stage before sodium dodecyl sulfate-phenol deproteinization, centrifuged, and fractions were analyzed by electrophoresis as in Figure 4. FDD-DNA is associated with a component of density 1.21–1.25 g/ml.

disease specific band was evident. We concluded that healthy plant protein was still present in these gradient fractions and that further purification will be required before FDD-DNA associated particles and proteins can be characterized.

DISCUSSION

The demonstration that FDD is transmitted by a single insect species (7) to MRD seedlings has permitted the development of an experimental system for testing whether a virus is the cause of the disease. We adopted an approach used previously for determining the cause of cadang-cadang (11) and lethal yellowing (10) diseases of coconut palm in that we looked for a disease-specific component in infected palms.

We have demonstrated a correlation between the presence of FDD symptoms and the detection of an unusual single-stranded DNA. The DNA was detected in both experimentally and naturally infected MRD plants. Although other unusual nucleic acid bands were found in some palms, their inconsistent isolation led to the conclusion that they were either artifacts of the extraction procedure or representative of microorganisms not specifically associated with FDD.

The detection of the DNA by extraction method A and preliminary indications of its relationship to FDD led to simplification of the method for its detection and allowed larger numbers of samples to be analyzed. These data further confirmed the association of the DNA with FDD and established that it can be used as a diagnostic marker for the disease, at least in MRD. The DNA was isolated in very small amounts and was only detectable in gels by silver staining. Diagnosis would appear to rely therefore on sensitive methods for detecting the DNA, based either on gel electrophoresis as described here, or on molecular hybridization.

The component with which FDD-DNA is associated has a maximum S value in sucrose density gradients close to that expected for members of the gemini-virus group (9). Small, single, but not double, spheres about 20 nm in diameter have occasionally been detected in extracts, and their association with FDD is to be further investigated. The buoyant density in Nycodenz of the component containing FDD-DNA falls in the range expected for virus nucleoprotein (4). Its density in Cs_2SO_4 is higher than that for the RNA containing bromoviruses, cucumoviruses and alfalfa mosaic virus, close to that determined for pea enation mosaic virus (5), and lower than that of pure DNA. These observations suggest that FDD-DNA is associated with other components and that it occurs in a distinct particle. Our inability to consistently associate a particle or specific protein with the FDD-DNA rich fractions suggest that such a particle may be either unstable under the conditions of transport, storage, and extraction or is unusual in its morphology.

The properties of the FDD-DNA established so far are not consistent with any known plant virus taxonomic group (9). The gemini viruses have circular single-stranded DNA in the molecular weight range $0.7–0.9 \times 10^6$, smaller than that currently estimated for FDD-DNA.

In summary, therefore, the detection of the disease-specific DNA is an indication that a virus is possibly the cause of FDD. It will be immediately useful as a diagnostic marker for FDD in coconut palm and should also be useful in the search for a particle. Establishment of clones of DNA fragments should allow the uniqueness of the FDD-DNA to be tested and allow development of an assay based on molecular hybridization. Such an assay would appear to be essential for determining the epidemiology and host range of FDD, thus allowing an assessment of the risk the disease presents to coconut improvement in the Pacific area.

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