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Transmission of the coconut cadang-cadang viroid to six species of palm by inoculation with nucleic acid extracts

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Seedlings of *Areca catechu* (betel nut palm), *Corypha elata* (buri palm), *Adonidia merrillii* (manila palm), *Elaeis guineensis* (oil palm), *Chrysalidocarpus lutescens* (palmera) and *Oreodoxa regia* (royal palm) were inoculated with nucleic acid extracts from coconut palms with cadang-cadang disease. Within 2 years of inoculation, analysis using a ^{32}P-labelled DNA probe complementary to the coconut cadang-cadang viroid (CCCV) showed that RNA sequences identical to CCCV were present in the inoculated seedlings. Electrophoresis in polyacrylamide gels showed that these palms also contained an RNA with mobility identical to CCCV. Four to five years after inoculation, the infected palms of four species were usually stunted compared with uninoculated palms, while betel nut and palmera were not stunted. Yellowing of leaflets was observed with defined spots or mottling of the older fronds in all except betel nut palms. All infected palms showed mild or severe yellow leaf spotting. These results widen the known host range and, hence, the potential number of viroid reservoir species in the field.

INTRODUCTION

The viroid associated with cadang-cadang infection of coconut (*Cocos nucifera* L.) (Randles, 1975) has been shown to cause the disease when inoculated to coconut palm (Zelazny *et al.*, 1982; Mohamed *et al.*, 1985). The smallest infectious unit of the coconut cadang-cadang viroid (CCCV) comprises 246 or 247 nucleotides (Haseloff *et al.*, 1982). In coconut, the viroid is present as a monomeric (CCCV-1) or dimeric (CCCV-2) form of the basic CCCV sequence. As the disease progresses in palms, molecular changes such as duplication of a segment in the molecule (Imperial *et al.*, 1981; Haseloff *et al.*, 1982) and addition of a cytosine residue (Imperial & Rodriguez, 1983) have been observed.

Several other palms species show cadang-cadang-like symptoms in the field (Bigornia, 1977) and detection of the viroid in diseased *Corypha elata* Roxb. (buri palm) and *Elaeis guineensis* Jacq. (oil palm) (Randles *et al.*, 1980) has shown that these species can be infected naturally. Since the mode of natural spread of cadang-cadang is unknown, various aspects of disease epidemiology are being examined. The presence of alternative plant reservoirs in the field is one important aspect and studies are in progress on the identification of additional species that are susceptible to CCCV.

This paper reports the experimental transmission of CCCV to seedlings of *Areca catechu* L. (betel nut), *Corypha elata*, *Adonidia merrillii* Becc. (manila palm), *Elaeis guineensis*, *Chrysalidocarpus lutescens* Wendl. (palmera) and *Oreodoxa regia* H.B. & K. (royal palm). Methods of inoculation and assay are described. The general appearance of infected plants is shown and the molecular forms of CCCV detected in the palms is described.

MATERIALS AND METHODS

Preparation of inoculum

Nucleic acids were extracted from leaves of infected coconut palms by blending chopped leaflets in 0·1 M Na_2SO_3 (3 ml/g leaflets) followed by the addition of 50 g polyethylene glycol (PEG) MW 8000 per litre of supernatant. Extraction of nucleic acids from the PEG pellet

was made by a series of treatments with a 2:1:1 mixture by volume of sodium dodecyl sulphate (SDS) (10 g/l): phenol (900 g/l): chloroform (Randles, 1975; Imperial *et al.*, 1981). Nucleic acids were recovered from the aqueous phase by precipitation with three volumes of ethanol. (All ethanol precipitations were made in the presence of at least 0·05 M sodium acetate.) The precipitate was either dried directly and dissolved in SSC (0·15 M NaCl and 0·015 M sodium citrate) or further purified by treatments with cetyltrimethylammoniumbromide (CTAB) and 2 M LiCl. SSC was used in all inocula.

Inoculation of seedlings

Seedlings were inoculated at the 2–3 leaf stage by high-pressure injection (Randles *et al.*, 1977) as shown in Fig. 1. More injections were given to the lower portion or the extreme base of the seedling. At least five injections (50 or 100 µl of inoculum per injection depending on the injector unit used) were given to each seedling. Nucleic acid extracted from approximately 100 g of coconut leaf was inoculated into each test plant. This gave an equivalent of around 0·1 or 0·4 mg of total nucleic acids with or without the CTAB-LiCl steps, respectively.

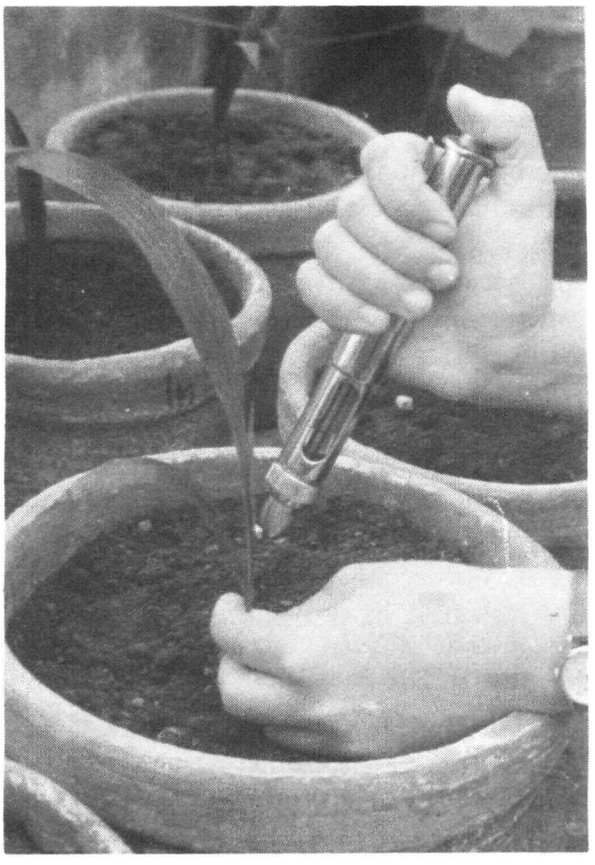

Fig. 1. Inoculation of an oil palm seedling by high-pressure injection of nucleic acid extracts. Injections were made at the base and leaflets were held in such a way that inoculum went through as many leaflets as possible including the youngest leaflet.

The seedlings were kept in the dark for at least 3 days before inoculation. The plants were either kept inside a screenhouse or planted in the field 2 months after inoculation.

CCCV assays

Molecular hybridization of dot-blotted extracts

Leaflets were obtained from the second or third youngest open frond. Total nucleic acids were extracted from the chopped leaflets using either of the following procedures. (1) 5-g samples were each blended in 15 ml of a 1:1 mixture by volume of phenol and AMES buffer (0·5 M sodium acetate buffer pH 6·0, 10 mM $MgCl_2$, 200 ml/l ethanol and 30 g/l SDS) (Laulhere & Rozier, 1976; M.W. Schwinghamer, personal communication). The homogenate was incubated at 37°C for 15 min and shaken with half its volume of chloroform. The nucleic acids were recovered from the supernatant by precipitation in CTAB (3·3 g/l) and the resulting pellet was washed twice with a 0·1 M sodium acetate solution in 75% ethanol. After drying, the precipitate was suspended in 0·1 mM EDTA or in 50 µl of a 0·01 M sodium acetate solution in 100 g/l sucrose (NAS). (2) 5-g samples were each blended in 15 ml of 0·1 M Na_2SO_3 and the nucleic acids in the supernatant were precipitated with CTAB. After one wash with the sodium acetate solution in ethanol the pellet was dried thoroughly and dissolved in NAS.

Molecular hybridizations to dot blots were done on nitrocellulose (Thomas, 1980) as previously described (Owens & Diener, 1981; Mohamed & Imperial, 1983; Symons, 1984). The probe used was a ^{32}P-labelled DNA complementary to CCCV which was generously supplied by J. Visvader and R. H. Symons of the University of Adelaide. This was synthesized from recombinant DNA containing full-length copies of CCCV.

Polyacrylamide gel electrophoresis (PAGE) of nucleic acid extracts

The extracts were prepared as for the dot-blot hybridization assay. These were purified further by treatment with 2 M LiCl for 16 h at 0°C and the nucleic acids were recovered from

the supernatant by ethanol precipitation. After drying, the precipitates were dissolved in NAS.

Electrophoresis was done in a 140 × 170 × 0·75 mm polyacrylamide gel (200 g acrylamide-bisacrylamide/l) (Imperial & Rodriguez, 1983) at 125 volts for 12 h in tris-borate-EDTA buffer (Peacock & Dingman, 1968). The gels were stained with silver as previously described (Sammons *et al.*, 1981) with the following modifications. Gels were fixed in 100 g/l trichloroacetic acid for 15 min followed by two brief rinses with water; they were soaked in 1·8 g/l $AgNO_3$ for 1 h; the reducing solution contained 0·25 M NaOH.

RESULTS

Molecular hybridization assay

Autoradiograms obtained for extracts from representative samples of the healthy and infected plants for each palm species are shown in Fig. 2. Extracts from known healthy and diseased coconut palms were included for reference. No hybridization with the ^{32}P-labelled probe was observed for any of the extracts from healthy plants while dark spots indicating a high degree of hybridization of the probe with CCCV in extracts from some inoculated palms

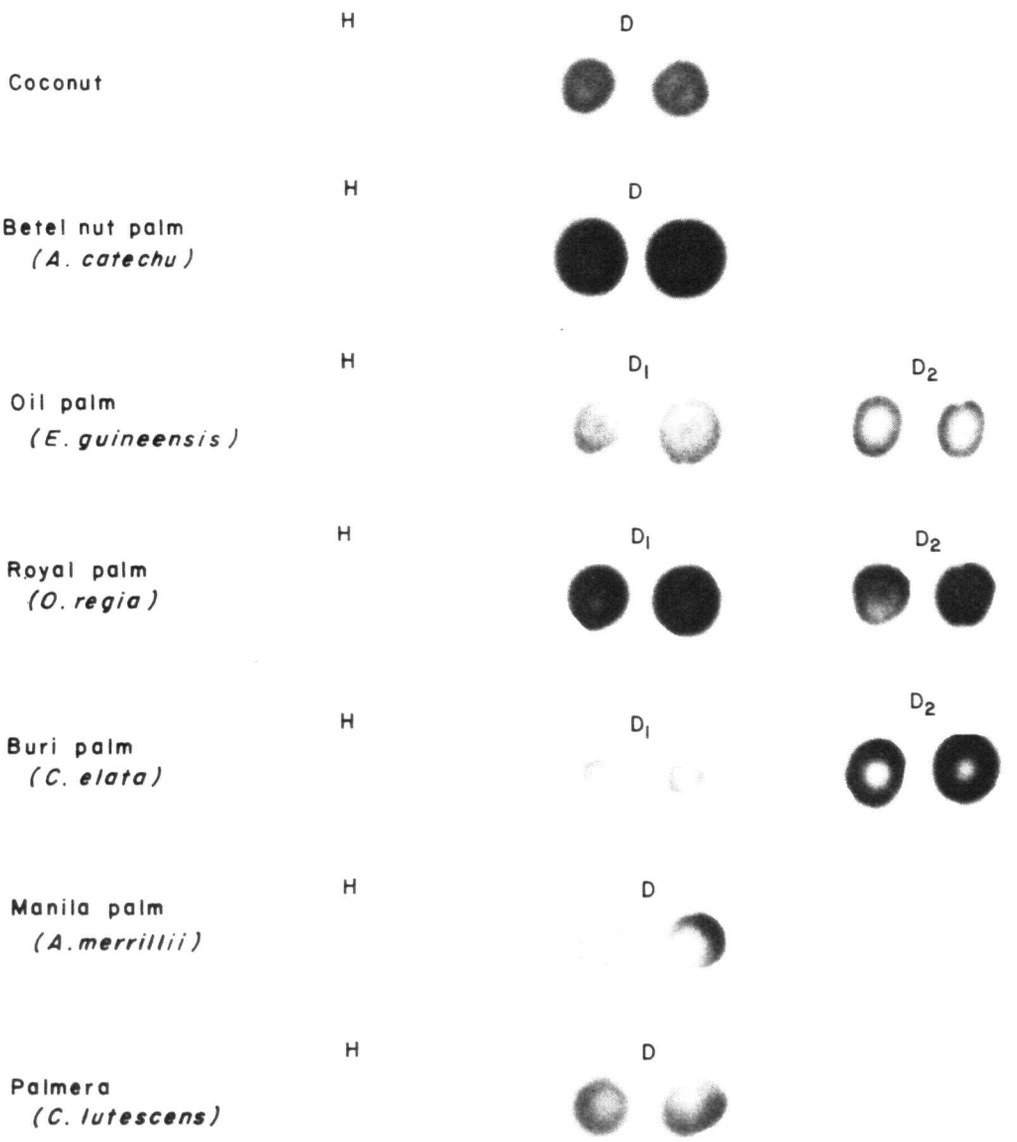

Fig. 2. Autoradiograms of extracts dot-blotted on cellulose nitrate sheets and hybridized with ^{32}P-labelled DNA complementary to CCCV. Extract from 0·1–0·2 g of leaf sample was used for each dot. H, from healthy leaf; D, from diseased leaf (D_1 and D_2 are from two different plants).

were proof of successful inoculation.

The transmission results obtained for each of the palm species are presented in Table 1. Three other palm species were inoculated but few survived and none of these were infected.

Gel electrophoresis (PAGE) assay

The polyacrylamide gel patterns obtained for the set of samples presented in Fig. 2 are shown in Fig. 3. Both fast (small) and slow (large) forms of CCCV were detected and these are also shown in Table 1. In this group of samples the slow form occurred in the 8-year-old palms and in some of the 5-year-old ones; only the fast form was detected in the younger palms (less than 5 years).

General appearance of infected plants

Within 2–3 years of inoculation, differences between infected and healthy plants were negligible. However, with few exceptions, varying degrees of stunting and leaf yellowing with defined spots or mottle in the older fronds were observed from the fourth year onwards. These are shown in Figs 4–9. Betel nut palm was an exception, and in Fig. 4 the 5-year-old infected palm was not markedly different from the healthy control in size or in the general appearance of the leaflets. The 5-year-old buri palm, however, showed severe stunting and yellowed leaflets compared to the uninoculated control (Fig. 5). A comparable difference between the inoculated positive and control palms was observed with the 8-year-old test plants of manila palm (Fig. 6). No stunting has yet been observed with the 2-year-old palmera (Fig. 7) but an appreciable yellowing of leaflets was apparent in the infected plants. A 5-year-old infected oil palm was stunted and yellowish compared to the control (Fig. 8). An 8-year-old infected royal palm in the first group of test plants was about two-thirds the height of the healthy controls of the same age. The yellowing of leaflets and presence of spots was well defined especially in the older fronds (Fig. 9).

Table 1. Transmission of coconut cadang-cadang viroid (CCCV) to six palm species. The test plants were inoculated at the 2–3 leaf stage, i.e. 3–8 months after germination. The assays were done by the dot-blot hybridization method alone or in combination with polyacrylamide gel electrophoresis (PAGE)

Palm species	No. of plants			CCCV		Average No. fronds per		
	Inoculated	At time of assay	Infected	First detection (years)	PAGE form	Infected palm	Healthy palm	Age (years) at growth assessment
Areca catechu Betel nut	7	5	4	2	Fast (D)[a]	8·3 (3[b])	11 (2)	5
Corypha elata	4	3	2	2	Fast	7·5 (2)	12 (1)	5
Buri palm	30	23	2	1·5	Fast (D_1) Slow (D_2)	8·5 (2)	8·3 (7)	2
Adonidia merrillii Manila palm	10	3	1	3	Slow (D)	5 (1)	13 (1)	8
Elaeis guineensis	9	6	4	2	Slow (D_2)	13 (3)	23 (8)	5
Oil palm	40	30	18	1·2	Fast (D_1)	6·5 (2)	11 (2)	3
Chrysalidocarpus lutescens	9	9	6	0·8	Fast (D)	13 (2)	13 (2)	2.5
Palmera	9	9	4	0·8	Fast	7·5 (2)	13 (2)	2.5
Oreodoxa regia	20[c]	20	2	3	Slow (D_2)	9 (1)	10·7 (3)	8
Royal palm	9	7	3	2	Fast (D_1)	5 (2)	6 (2)	5

[a]As shown in Figs 2 and 3. PAGE and growth assessment were done simultaneously.
[b]Number of palms.
[c]Inoculated by G. Boccardo.

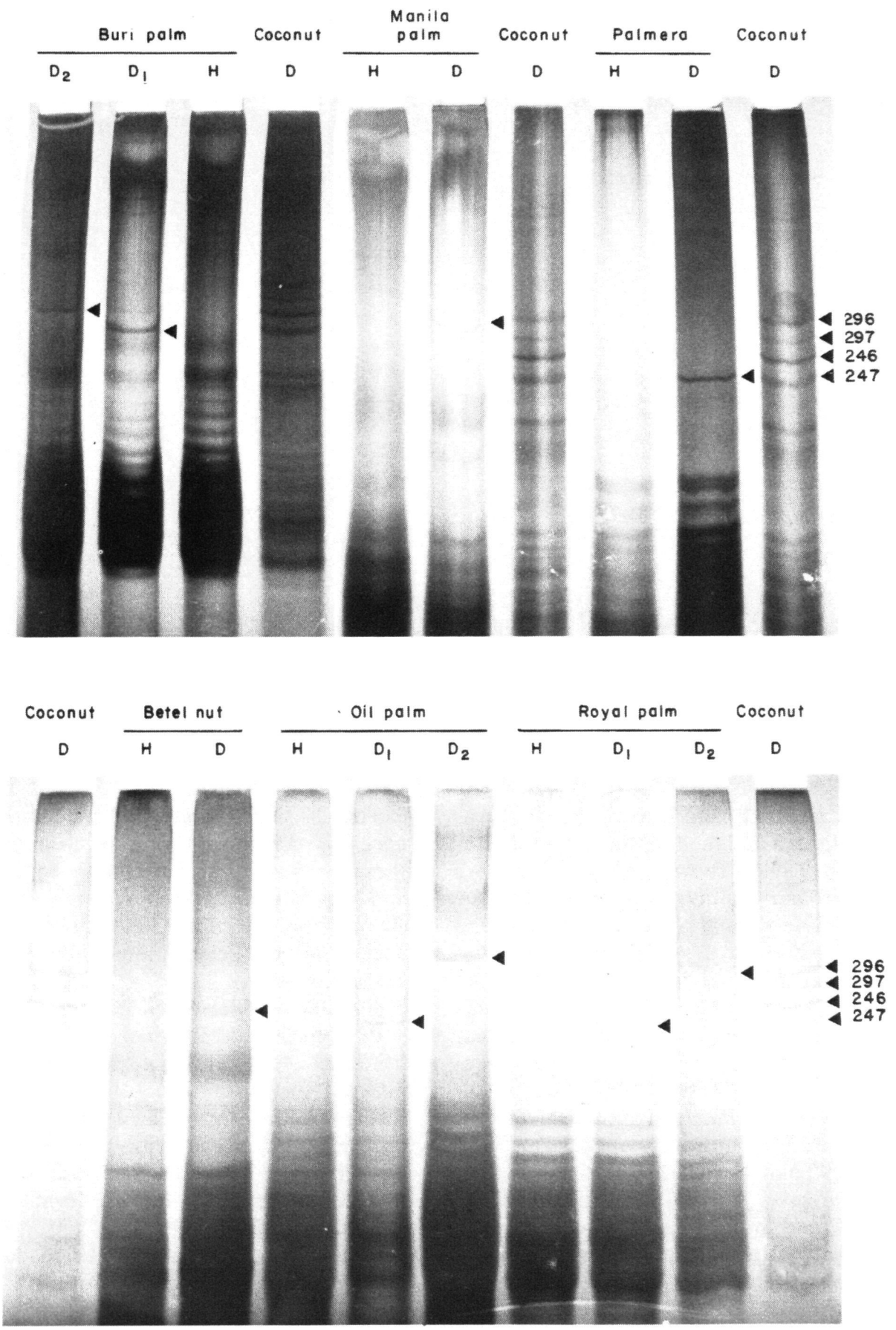

Fig. 3. Electrophoresis patterns in polyacrylamide gels after staining with silver. Extracts from 1–3 g of leaf sample were applied in each lane. The marker CCCV from coconut palm contains the four known monomeric forms of CCCV each marked by an arrow and the estimated number of base residues (Haseloff *et al.*, 1982; Imperial & Rodriguez, 1983). H, from healthy leaf; D, from diseased leaf (D_1 and D_2 are from two different plants). Identical plants were used for Figs 2 and 3.

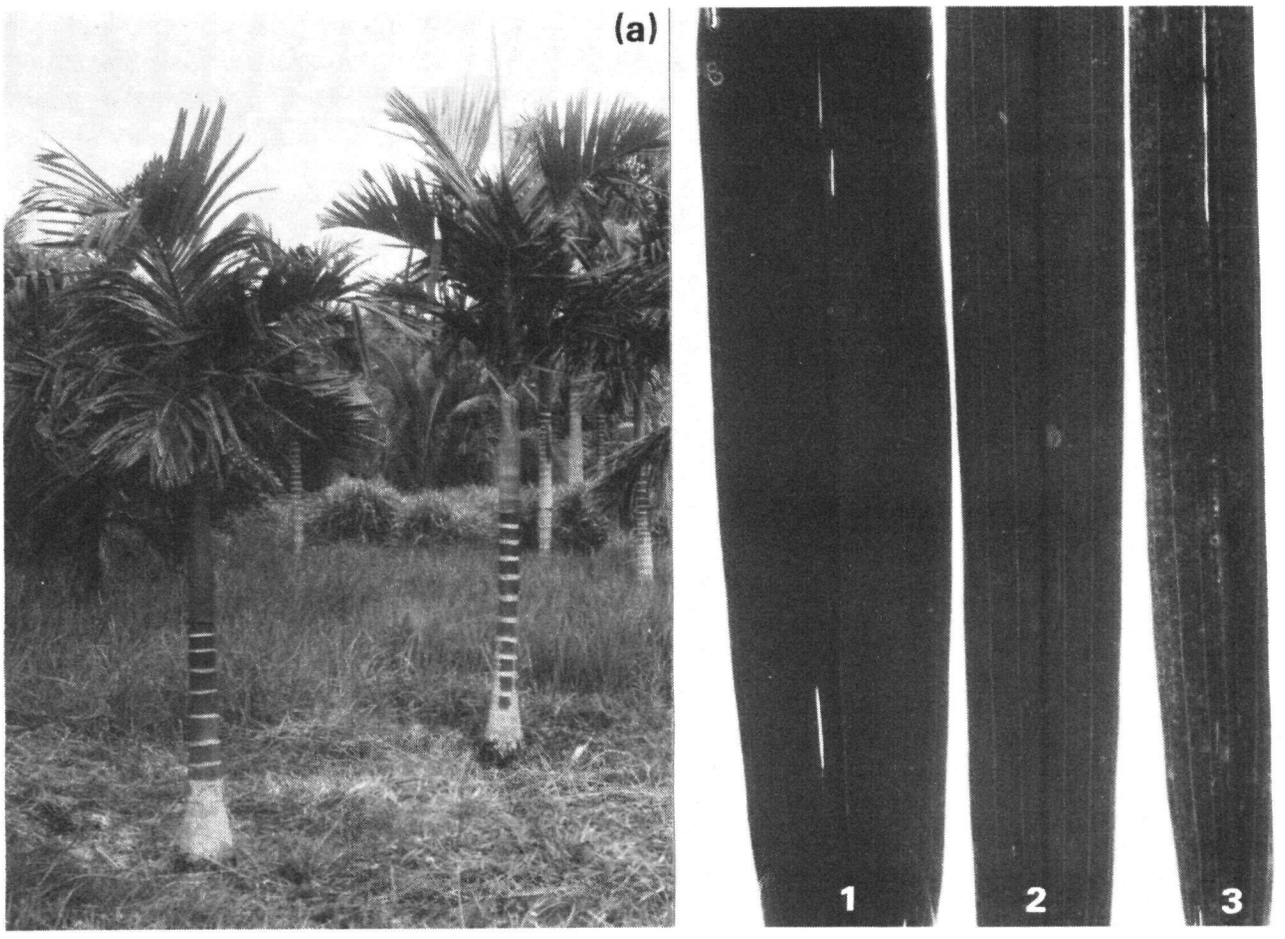

Fig. 4. (a) A 5-year-old infected betel nut palm (left) and a healthy control. (b) 1, leaflet from the sixth frond of the healthy palm; 2, leaflet from the third frond of the infected palm; 3, leaflet from the sixth frond of the infected palm.

The other infected palm in the same trial was severely stunted and died in the fifth year after inoculation. Table 1 shows mean frond number for infected and healthy plants as an indicator of comparative growth. (See data for coconut palm in Randles *et al.*, 1977.)

A test was made to determine whether the leaf spots observed in the older infected plants were similar to the typical cadang-cadang leaf spots in coconut (Zelazny *et al.*, 1982). Surface-sterilized leaf strips were incubated on potato dextrose agar and bacterial or fungal growth was observed from most of the leaf strips from the infected palm species. The infected coconut leaf strips gave minimal growth of micro-organisms, and in most cases none at all. The pathogenicity of the isolated micro-organisms was not tested.

DISCUSSION

The recovery of CCCV from seedlings of betel nut, buri, manila, palmera, oil and royal palms, inoculated up to 3 years previously with nucleic acid extracts from coconut leaves with cadang-cadang disease, has provided unequivocal evidence for the transmission of the viroid into these palm species. Differences in symptoms and growth indicated different reactions of the six species to infection. Continued evaluation of these palms and inoculation of a larger number of seedlings will allow a more quantitative evaluation of differences in symptomatology.

Unlike coconut, in which the association of bacteria or fungi with leaf spots is minimal, the infected plants of most of the above palm species frequently seemed to have micro-organisms associated with the leaf spots. If further work shows that these are pathogenic, they could enhance palm deterioration following CCCV infection.

Further investigations will be made on the occurrence of fast and slow forms of CCCV in the infected palms. The preliminary observation suggesting that there is a direct correlation between frequency of detecting slow CCCV and length of time after inoculation in some palms indicates that the molecular transition observed in coconut may also occur in these palms.

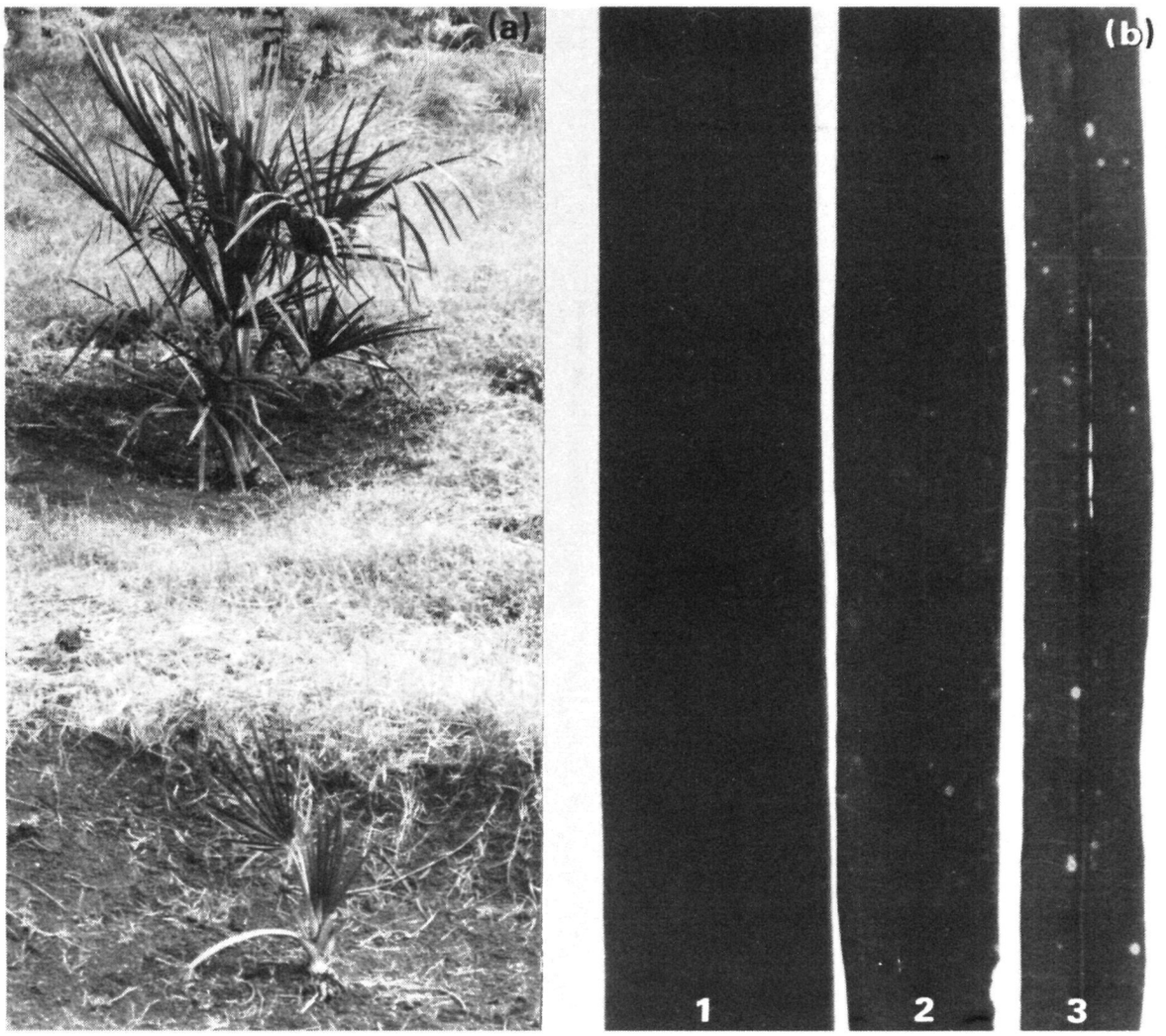

Fig. 5. (a) A 5-year-old infected buri palm (foreground) and a healthy buri palm of the same age. (b) 1, leaflet from the third frond of the healthy palm; 2 and 3, leaflets from the third frond of the infected palm.

Current work on the host range of CCCV includes inoculation of other palm species such as date (*Phoenix dactylifera*), anahaw (*Livistona rotundifolia*) and macarthur (*Actinophloeus macarthuri*) palms, and inoculation of coconut sprouts with extracts from other infected palm species. Attempts are also being made to transmit the viroid experimentally to non-palm plant species. These experiments have the objective of identifying the plant species (palm or non-palm) that should be monitored in the field for any possible role in the spread of the cadang-cadang pathogen.

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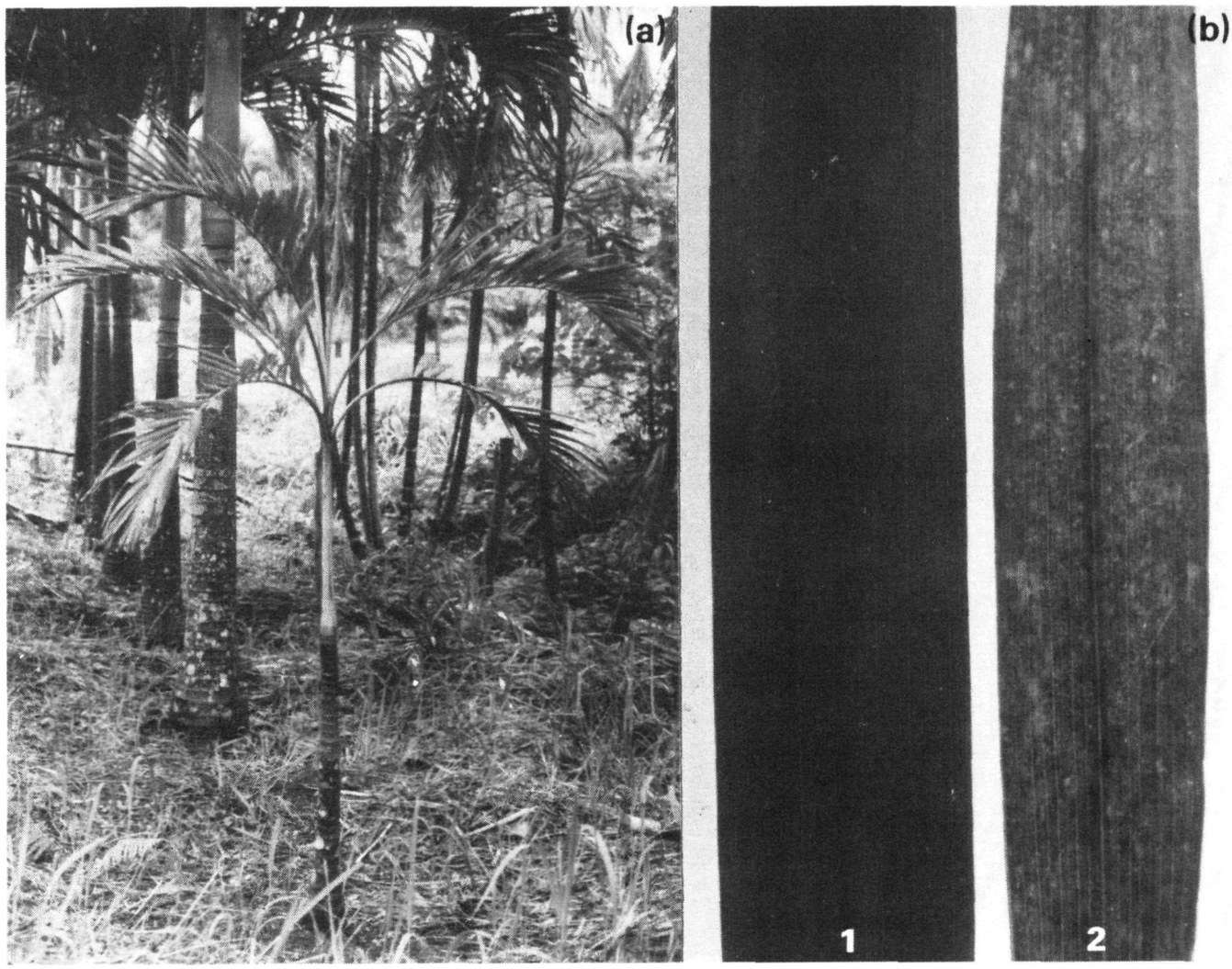

Fig. 6. (a) An 8-year-old infected manila palm and a row of healthy 8-year-old manila palms in the background. (b) 1, leaflet from the third frond of a healthy palm; 2, leaflet from the third frond of the infected palm.

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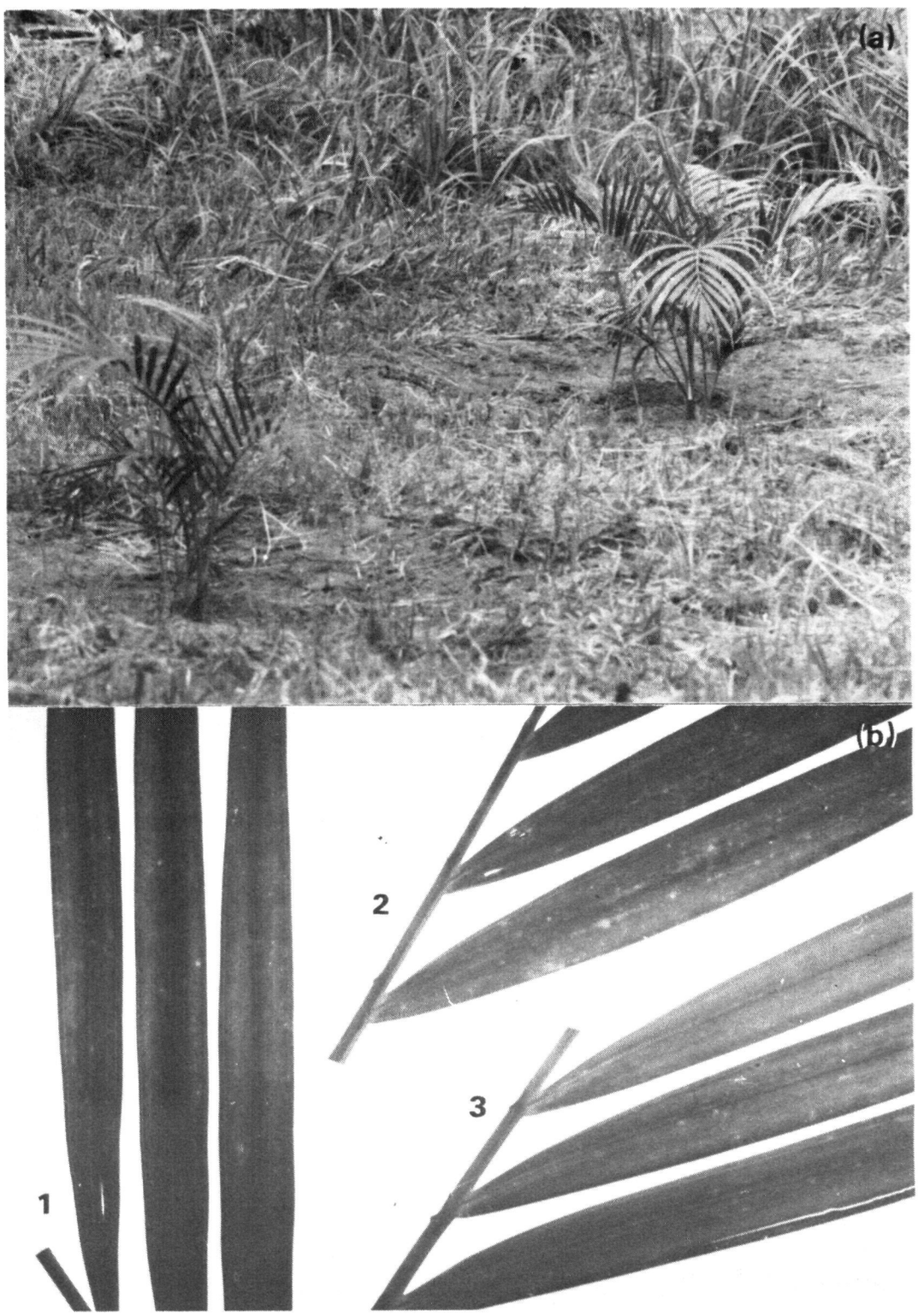

Fig. 7. (a) A 2-year-old infected palmera (right) and a healthy control. (b) 1, leaflets from the third frond of the healthy palmera; 2 and 3, leaflets from the third frond of two infected palmeras.

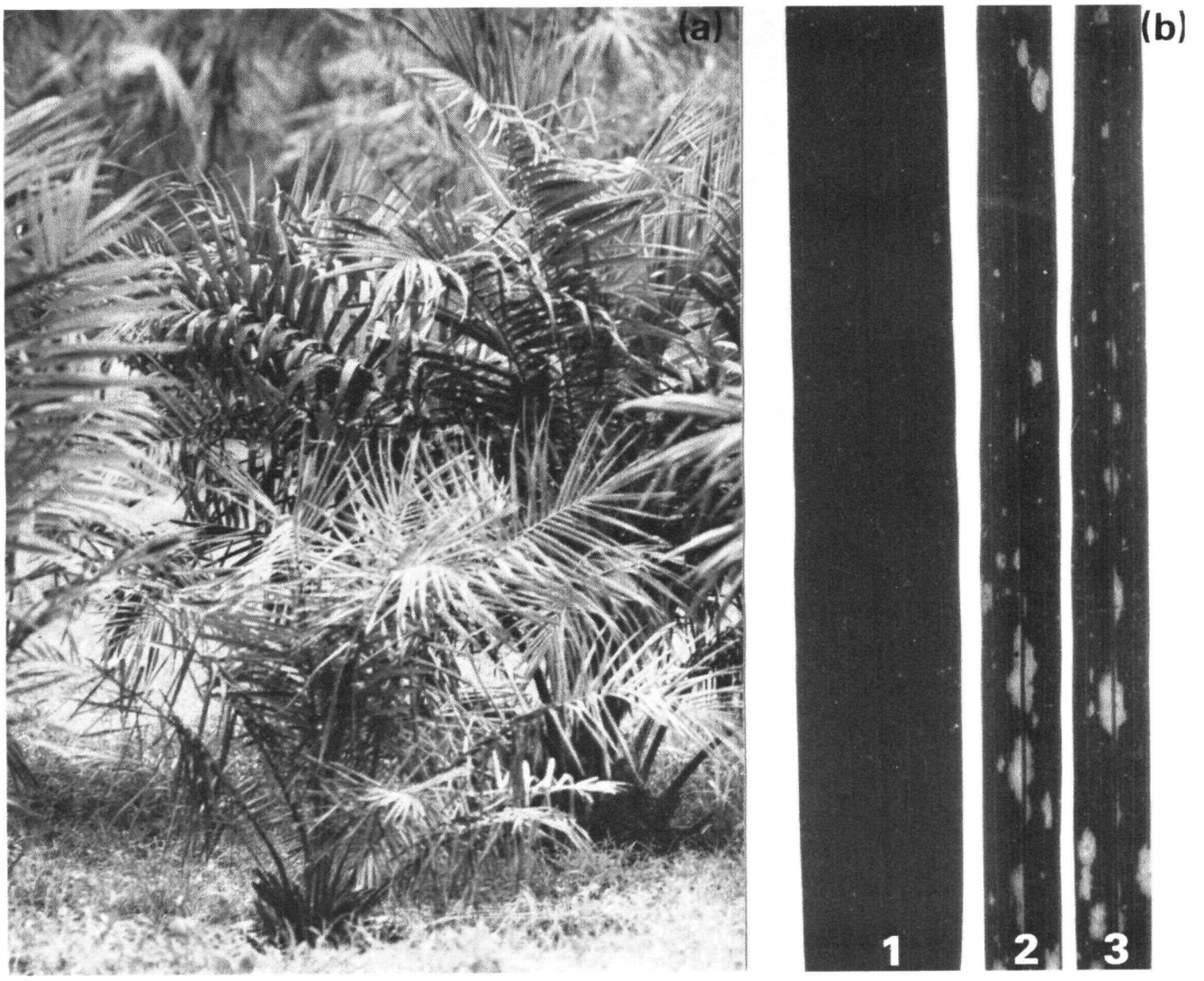

Fig. 8. (a) A 5-year-old infected oil palm and a healthy 5-year-old oil palm (background). (b) 1, leaflet from the sixth frond of the healthy palm; 2 and 3, leaflets from the sixth frond of the infected palm.

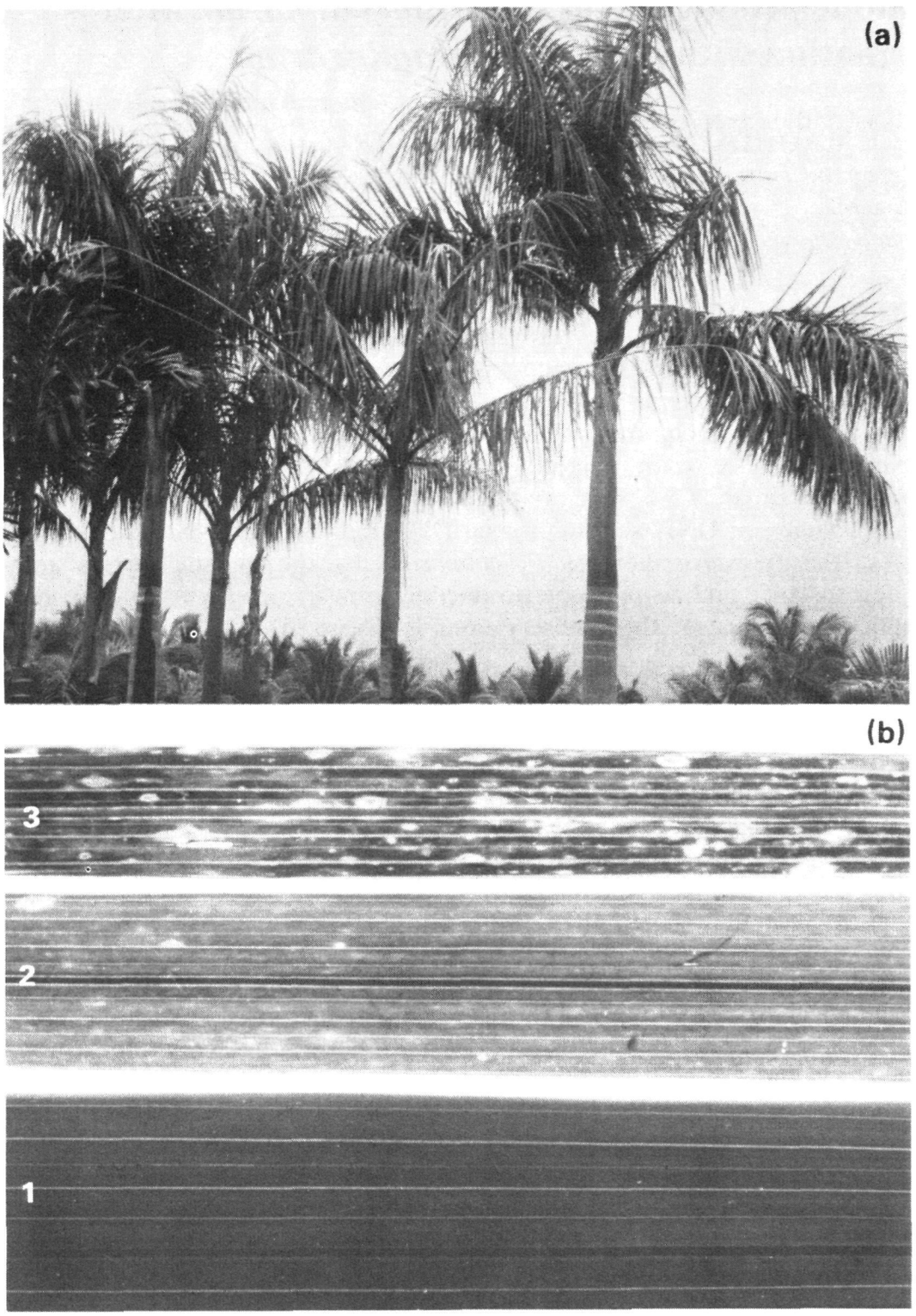

Fig. 9. (a) An 8-year-old infected royal palm (centre) and healthy royal palms of the same age. (b) 1, leaflet from the sixth frond of a healthy royal palm; 2, leaflet from the third frond of the infected palm; 3, leaflet from the sixth frond of the infected palm.