# Purification and Infectivity of the Coconut Cadang-cadang Viroid

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## ABSTRACT

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A procedure is described for the extraction of cadang-cadang viroid RNAs (cc RNA) from infected coconut leaves and their purification by three cycles of polyacrylamide gel electrophoresis. The fast and slow forms of cc RNA-1 (246 and 287 residues, respectively) and the fast form of cc RNA-2 (492 residues) were all infectious. There was no significant difference between the infectivities of the fast forms of cc RNA-1 and -2 and between linear and circular forms of cc RNA-1. The fast form of cc RNA-1 (246 residues) was significantly more infectious ($P < 0.001$) than the slow form (287 residues) from the same palm; the extra repeated segment of 41 nucleotide residues reduced infectivity of the cc RNA significantly. Therefore, the fast form appears to be the basic infectious unit of the cadang-cadang viroid RNAs and is designated to be the coconut cadang-cadang viroid.

Two circular viroidlike RNA species, cc RNA-1 and cc RNA-2, are associated with the cadang-cadang disease of coconut palms (8,2). The two RNA species occur in infected palms as fast and slow electrophoretic forms (5), the fast form occurring during early stages of disease while the slow form is found at later stages (3). The fast form of cc RNA-1 consists of 246 or 247 residues and the slow form, which contains an extra repeated segment of the fast form, consists of 287-301 residues, the actual length depending on the isolate (2). The cc RNA-2 forms are dimers of the corresponding cc RNA-1 forms (2). The role played by these different RNA species in infectivity is not known although partially purified nucleic acid extracts containing a mixture of cc RNAs and higher molecular weight nucleic acids have been shown to be infectious (9,5). However, this is not sufficient evidence for a viroid etiology especially in view of reports (10) that viroidlike RNA can be encapsidated in virus particles.

In this paper, we describe a method based on a combination of methods for small-scale extractions of cc RNAs (5,11) for large-scale purification of the viroid RNAs and for purification of chrysanthemum stunt viroid (6). The infectivities of the purified cc RNAs are demonstrated and their relative infectivities compared.

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## MATERIALS AND METHODS

**Source of infected material.** Coconut palms naturally infected with the cadang-cadang viroid were used as a source of tissue; fronds 6 (5–6 mo old) to 20 (18 mo old) were used as these contained

the highest concentration of cc RNAs (4). Approximately 1 kg of leaflets were obtained from each frond. Leaflets were stripped from the petiole, mid-ribs were removed and the leaf blades were chopped into 1-cm square pieces with a chaff cutter.

**Extraction of nucleic acids.** Nucleic acids were extracted from the leaves by a method based on a previously described procedure (5). Batches (250 g) of chopped leaves were blended in 750 ml of unbuffered 0.1 M sodium sulphite. The slurry was filtered and clarified by centrifugation at 10,000 *g* for 10 min. Supernatants from 5 kg of leaf material were pooled and polyethylene glycol 6000 was added to precipitate the macromolecules. The pellets were collected by centrifugation and extracted twice with SDS-phenol-chloroform; nucleic acids were precipitated from the aqueous phase by the addition of three volumes of ethanol. The resulting pellet was collected by centrifugation and reextracted with SDS-phenol-chloroform. Nucleic acids were collected from the aqueous phase by ethanol precipitation. They were then resuspended in 0.1 M sodium acetate and one-half volume of 1% cetyl trimethyl ammonium bromide was added to reprecipitate the nucleic acids. This pellet was collected by centrifugation and washed four times with 75% ethanol containing 0.1 M sodium acetate. The pellet was then resuspended in 0.1 M sodium acetate and single-stranded nucleic acids were removed by LiCl treatment; the remaining nucleic acids (including the viroid RNAs) were precipitated by the addition of three volumes of ethanol. The final nucleic acid pellets were resuspended in 0.01 M sodium acetate, 5% sucrose, for electrophoresis.

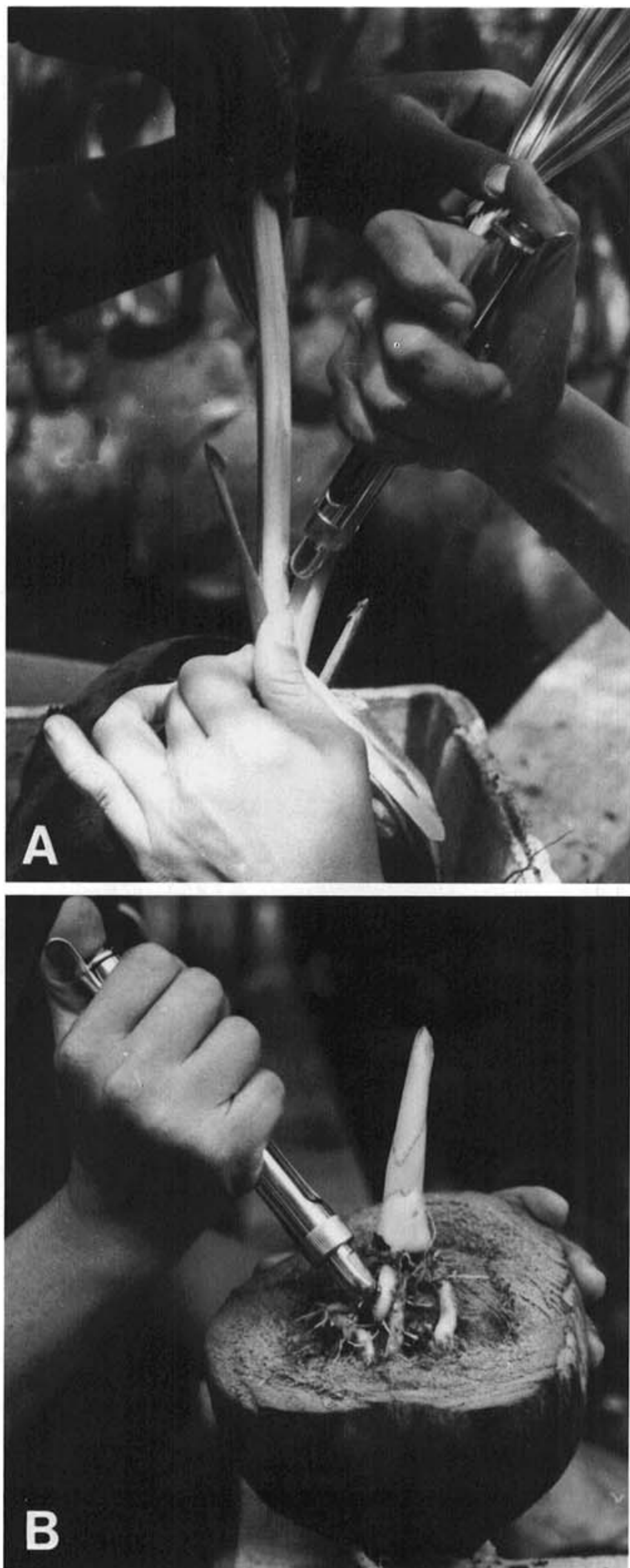

**Fig. 1.** Inoculation of coconut seedlings with a Panjet. **A,** Three-mo-old seedling. **B,** Newly sprouted seedling.

**Fractionation of nucleic acids.** The cc RNAs were separated from plant nucleic acids by three cycles of polyacrylamide gel electrophoresis (PAGE) using slab gels (6). In the first cycle, total nucleic acid extracts from leaves were electrophoresed under nondenaturing conditions in 3-mm-thick 5% gels in tris-borate-EDTA (TBE) buffer (7). After staining with toluidine blue, gel slices containing the required RNAs were cut out. Each slice was layered on 3-mm-thick 5% gels containing 8 M urea in TBE buffer; the RNA band migrated into the main gel during electrophoresis. After electrophoresis, the linear and circular forms (6) were located by staining and gel slices containing these forms were excised. Each slice was layered on 3-mm-thick 3.3% gels in TBE buffer and, after electrophoresis, the RNA band was recovered by elution from the base of the gel by using a Bio-Rad preparative apparatus. The RNA was collected by precipitation with three volumes of ethanol containing 0.1 M sodium acetate.

**Inoculation of coconut seedlings.** Coconut (*Cocos nucifera* L. 'Tambolilid') seednuts were collected from 100 randomly selected 25- to 30-yr-old open-pollinated palms; the same palms provided seednuts for all experiments. The seedlings were inoculated by high-pressure injection with a Panjet (Schuhco International, London, UK) (9). In method A, 3-mo-old seedlings were kept in the dark for 24 hr prior to inoculation by injection with four 100-$\mu$l doses into the base of the spear leaf (Fig. 1a). Seedlings were then kept under shade for 2–3 wk before transplanting to the field at 2-m spacings. In method B, seednuts were partially dehusked, as soon as the plumule began to emerge from the husk, thus exposing the roots and base of the shoot (Fig. 1b). The seedlings were inoculated by injection with a Panjet delivering 25 $\mu$l per shot; four doses were applied to the base of the plumule and four to the roots. The inoculated seedlings were kept in moist sand in the shade for 3–4 wk, transplanted to soil in polythene bags and kept in a seed garden for 2–3 mo, then transplanted to the field.

**Assay of inoculated seedlings.** Inoculated seedlings were analyzed for the presence of diagnostic cc RNAs 1–2 yr after inoculation by taking 10-g samples from 3-mo-old fronds and extracting nucleic acids for electrophoresis on 5% gels (5).

## RESULTS

**Purification of cc RNAs.** In the first cycle of PAGE, electrophoresis of the nucleic acid extracts on a 5% nondenaturing gel separated the fast and slow forms of cc RNA-1 and -2 from host nucleic acids (Fig. 2a). During the second cycle of PAGE under denaturing conditions, the circular and linear forms of the cc RNAs were separated (Fig. 2b) from each other and from host single-stranded RNAs. It was thus possible to obtain the circular form free of detectable contamination with other nucleic acids. After the final cycle, electrophoresis under nondenaturing conditions, the viroid RNAs were recovered from the base of the gel by elution; most of the RNA was collected in 3–4 ml of eluant.

Average yields of purified cc RNAs obtained from 35 palms are shown in Table 1. Comparable yields of the fast and slow forms were obtained from different palms and from separate fronds of the same palm. Yields of the linear forms of cc RNA-1 were between 5 and 10% of the corresponding circular forms. Yields of fast cc RNA-2 were about 10% those of the fast cc RNA-1 form, but yields of slow cc RNA-2 were less than 5% those of the slow cc RNA-1.

**Purity of the isolated cc RNAs.** Fast and slow forms of cc RNA were purified from a palm in the early stage of disease from fronds containing both forms (3). Electrophoresis of the individual purified RNAs on 5% analytical gels demonstrated that each of the fast and slow forms of cc RNA-1 and -2 were obtained free of detectable amounts of the other forms (Fig. 2c). Loading of gels with up to ten times (ie, 10 $\mu$g instead of 1 $\mu$g) the usual amounts of RNA failed to show the presence of any contaminants. As the fast and slow forms were usually purified from different palms or fronds that contained only one form of cc RNA, the likelihood of cross-contamination was therefore minimal.

Two-dimensional fingerprints of ribonuclease A and $T_1$ digests of purified circular cc RNA-1 were prepared as previously described (3); the results showed that the number of fragments and patterns obtained were consistent with the molecular complexity of the RNA (2). This indicated that there was no detectable contamination of the cc RNA preparations with nonviroid RNAs.

The thermal denaturation profiles of purified circular cc RNA (in 15 mM sodium chloride, 1.5 mM sodium acetate, pH 7.0) had a sharp thermal transition which is characteristic of viroids (1); the $T_m$ was 55 C and the percentage of hyperchromacity was 20%. The UV spectrum of purified cc RNA was typical of nucleic acids with a maximum at 258 nm and a minimum at 236 nm; the $A_{260\ nm} : A_{280\ nm}$ ratios were in the range from 2.10 to 2.40.

**Infectivity of the cc RNAs.** The results of infectivity trials for the circular forms of the cc RNAs are summarized in Table 2 and show that fast cc RNA-1 and -2 and slow cc RNA-1 were all infectious. Although no infectivity was associated with slow cc RNA-2, this was probably due to the small number of seedlings that were inoculated because it was difficult to purify sufficient amounts of this form.

When the fast forms of the viroid RNAs were used as inoculum, fast cc RNA-1 and -2 were the first forms to be detected in infected seedlings (Table 2); the slow forms were detected in newly developed fronds of these seedlings about 1 yr later. When the slow form of cc RNA-1 was used as inoculum, four of six infected seedlings were found to contain the slow form at the initial sampling. Of the remaining seedlings, one contained both fast and slow forms in the frond sampled while in the other seedling the

TABLE 1. Average yields of purified coconut cadang-cadang viroid RNAs (cc RNA) obtained from naturally infected palms

| Type of cc RNA | Form | Yields of cc RNA ($\mu$g/kg) | |
|---|---|---|---|
| | | Average | Range |
| Fast cc RNA-1 | | | |
| (246-247 residues) | Circular | 68 | (23–154) |
| | Linear | 5 | |
| Slow cc RNA-1 | | | |
| (287-301 residues) | Circular | 77 | (25–280) |
| | Linear | 8 | (3–13) |
| Fast cc RNA-2 | | | |
| (492-494 residues) | Circular | 8 | (1–25) |
| Slow cc RNA-2 | | | |
| (574 residues) | Circular | 4 | (1–5) |

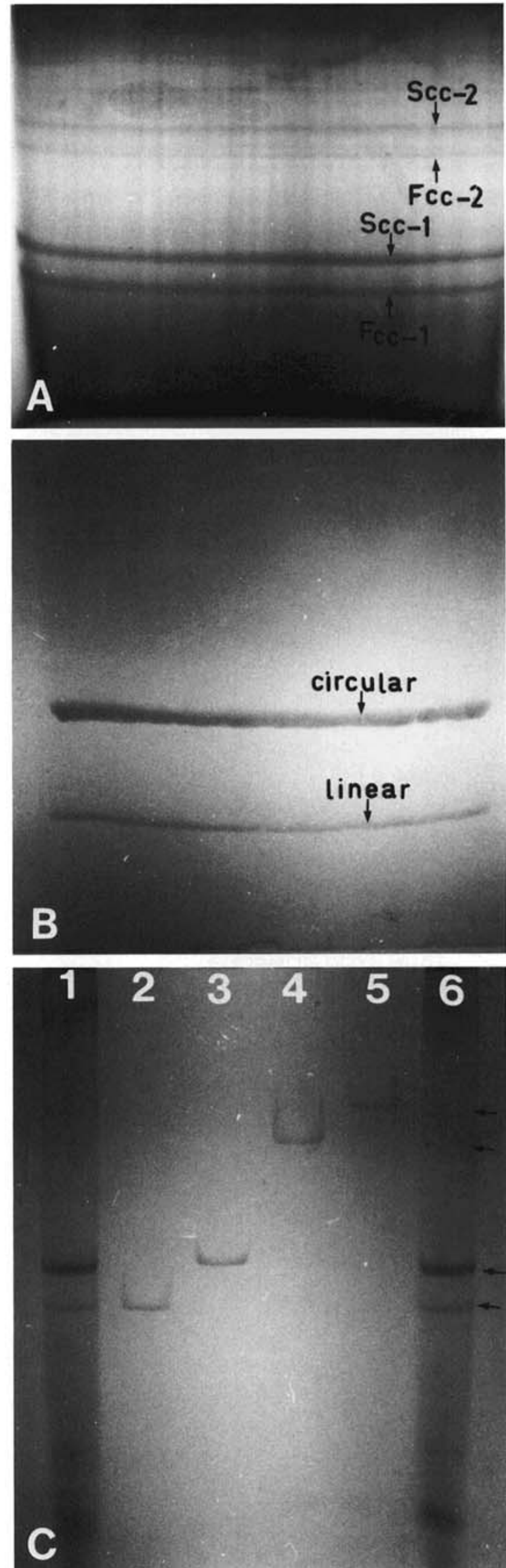


**Fig. 2. A,** Separation of coconut leaf nucleic acid extracts, from an infected palm containing fast and slow forms of coconut cadang-cadang viroid RNA (cc RNA), on a 5% preparative polyacrylamide gel under nondenaturing conditions. Electrophoresis was for 2.5 hr at 40 mA/gel. **B,** Separation of the linear and circular forms of slow cc RNA-1 on a 5% polyacrylamide gel under denaturing conditions (tris-borate-EDTA buffer containing 8 M urea). Electrophoresis was for 4 hr, 40 mA/gel, at 40–50 C. **C,** Electrophoresis of preparations of fast and slow forms of cc RNA-1 and -2 purified from the same palm. Electrophoresis was for 2.5 hr at 25 mA on a 5% gel. Lanes 1 and 6: total leaf nucleic acids from an early-stage diseased palm; lane 5: slow cc RNA-2; lane 4: fast cc RNA-2; lane 3: slow cc RNA-1; lane 2: fast cc RNA-1.

older (3-mo-old) fronds contained the fast form and the younger (1-mo-old) fronds had both forms.

It was not possible to establish any clear relationship between inoculum concentrations and rate of infection in most experiments as levels of infection were low (<10%). However, in one experiment (Table 3), where the average rate of infection was 68%, the dose-response curve was flat over the range of concentrations tested.

**Relative infectivities of the viroid RNAs.** The infectivities of the fast forms of cc RNA-1 and -2 were compared as in naturally infected and mechanically inoculated palms; these forms are the first to be detected. These RNAs were isolated from the same palms and inoculated to test seedlings separately and together. The results showed that there was no significant difference between infection rates for fast cc RNA-1 (10 infected/134 inoculated) and fast cc RNA-2 (8/131). The infection rates for the two forms inoculated together (9/99) and separately (10/98) were also not significantly different indicating that there was no interaction between the two forms.

Infectivities of fast and slow forms of cc RNA-1 were compared as these are present in infected palms at different stages of disease. Earlier experiments with partially purified extracts from different palms had indicated that the fast form was more infectious (5). The results of these experiments were confirmed by using purified RNAs from different palms (Table 4); circular fast cc RNA-1 was significantly more infectious than circular slow cc RNA-1 (9 positive out of 10 inoculated, $P = 0.01$).

In a second experiment, the two forms of cc RNA-1 were purified from different fronds of the same palm in the early stage of disease (3); the fast form was obtained from fronds 12–17 and the slow form from fronds 3–7. The nucleotide sequences of the viroid RNAs from this palm (Baao #54) have been determined (2); therefore, it was possible to compare the infectivities of two RNA species whose nucleotide sequences were known. The cc RNAs were inoculated in equimolar ratios (Table 3) (ie, in proportion to the relative molecular sizes of the two forms) 246 residues for fast cc RNA-1 and 287 residues for slow cc RNA-1. Newly sprouted seedlings were inoculated by method B in October 1981 and analyzed for presence of cc RNAs in June 1983. The results (Table 3) show that the fast form of cc RNA-1 is significantly more infectious ($P < 0.001$) than the slow form from the same palm.

The infectivities of the circular and linear forms of fast cc RNA-1 were compared by purifying the two RNAs from the same fronds of a palm in the early stage of disease and inoculating 10 seedlings with each form at concentrations of 25 and 5 μg/ml. The infection rates for the two forms were comparable, 3/20 infected seedlings for the circular form and 2/20 for the linear form.

**Comparison of infection rates for 3-mo-old and newly sprouted seedlings.** The efficiencies of inoculation method A (with 3-mo-old seedlings) and method B (newly sprouted seedlings) were compared by analyzing results of experiments in which the same inoculation source had been used to inoculate seedlings by the two methods. The results (Table 5) show that method B gave consistently higher rates of infection and that this method was, overall, more efficient than method A ($P < 0.001$). This indicates that the younger seedlings were more susceptible to infection than the older ones.

## DISCUSSION

The methods described in this paper for the extraction and purification of the cc RNAs have made it possible to obtain relatively pure preparations of the different forms of cc RNA in quantities sufficient for infectivity studies. The yields of circular cc

TABLE 2. Relative infectivities of the coconut cadang-cadang viroid RNAs (cc RNA)

| Inoculum[a] | Concentration[b] (μg/ml) | No. of plants:[c] Inoculated | Infected | cc RNA form first detected[d] |
|---|---|---|---|---|
| Fast cc RNA-1 | 100 - 0.01 | 270 | 57 | All fast |
| Fast cc RNA-2 | 50 - 0.002 | 131 | 8 | All fast |
| Slow cc RNA-1 | 180 - 0.01 | 182 | 6 | 4 - Slow<br>1 - Fast + slow<br>1 - Fast |
| Slow cc RNA-2 | 25 | 10 | 0 | |
| Controls[e] | | 155 | 0 | |

[a]Purified cc RNAs were used for inoculation.
[b]Figures are a compilation of results of injections made over the range of concentrations shown.
[c]Number of inoculated seedlings in which diagnostic cc RNAs were detected.
[d]Form of cc RNA detected in infected seedling at initial sampling.
[e]Inoculated with buffer only.

TABLE 3. Relative infectivities of the fast and slow forms of coconut cadang-cadang viroid RNA-1 (cc RNA-1) isolated from coconut palm Baao #54

| Experiment | Fast cc RNA-1 (246 residues) Concentration[a] | Infectivity[b] | Slow cc RNA-1 (287 residues) Concentration[a] | Infectivity[b] |
|---|---|---|---|---|
| 1 | 80 | 7/9 | 96 | 2/9 |
| 2 | 20 | 6/9 | 24 | 0/9 |
| 3 | 10 | 7/10 | 12 | 0/10 |
| 4 | 5 | 7/10 | 6 | 0/10 |
| 5 | 2.5 | 5/9 | 3 | 0/8 |

$d = 0.639 \pm 0.194$ (95% confidence limits)
Test H : $d = 0$, $Z = 6.46$ ($P < 0.01$)

[a]Concentration of RNA in the inoculum (μg/ml).
[b]Infectivity expressed as the number of infected seedlings over the number of seedlings inoculated.

TABLE 4. Relative infectivities of the fast and slow forms of coconut cadang-cadang viroid RNA-1 (cc RNA-1) purified from different palms

| Experiment | Concentration of cc RNA-1 (μg/ml) | Percentage[a] of seedlings infected on inoculation with: Fast cc RNA-1 | Slow cc RNA-1 | Sign[b] test |
|---|---|---|---|---|
| 1 | 100 | 50 | 0 | + |
| 2 | 64 | 10 | 0 | + |
| 3 | 25 | 10 | 0 | + |
| 4 | 25 | 10 | 0 | + |
| 5 | 10 | 20 | 0 | + |
| 6 | 6.4 | 40 | 50 | – |
| 7 | 5 | 10 | 0 | + |
| 8 | 5 | 10 | 0 | + |
| 9 | 5 | 20 | 0 | + |
| 10 | 0.1 | 10 | 0 | + |

[a]Ten seedlings were inoculated in each experiment.
[b]Null hypothesis that there was no difference in the infectivities of the two forms.

TABLE 5. Comparison of the rate of infection of young and 3-mo-old coconut seedlings

| Inoculum source[a] | Form of cc RNA in inoculum | Method of inoculation[b,c] A: Inoculated (no.) | A: Infected (no.) | B: Inoculated (no.) | B: Infected (no.) |
|---|---|---|---|---|---|
| $B_2$ | Fast cc RNA-1 | 50 | 2 | 34 | 5 |
| | Fast cc RNA-2 | 47 | 1 | 35 | 3 |
| $B_5$ | Slow cc RNA-1 | 50 | 0 | 21 | 4 |
| $B_2 + B_5$ | Fast + slow cc RNA-1 | 38 | 2 | 23 | 12 |

$d = 0.1898 \pm 0.06797$ (95% confidence limits)
Test H : $d = 0$, $Z = 5.475$ ($P < 0.001$)

[a]Denotes palm from which inoculum was purified.
[b]Represents pooled results for a range of inoculum concentrations.
[c]Method A—Base of the spear leaf of 3-mo-old seedlings injected with four 100-μl doses. Method B—Base of the shoot (exposed by partially dehusking a germinating seednut) injected with four 25 μl doses.

RNA-1 (70 μg/kg) are comparable to those obtained with other viroids (1).

The infectivity of the viroid RNAs has been demonstrated by the detection of diagnostic cc RNAs in inoculated seedlings. Only a few of these infected seedlings are showing early leaf symptoms at this stage (bright yellow spots on young leaves). The diagnostic fruit and leaf symptoms (3) cannot be expected to appear for another 5–10 yr. However, the presence of the diagnostic cc RNAs in inoculated seedlings can be accepted as proof of successful transmission (9).

The results of experiments with inocula from different palms indicates that there is considerable variation between rates of infection with different inocula. Therefore, relative rates of infection of the various cc RNA forms could only be compared within experiments (using the same inoculum) and not between experiments. The problem was compounded by the low rates of infection (5–10%) obtained in most experiments. At a rate of infection of 5%, over 400 seedlings would need to be inoculated with each treatment to detect a doubling in infection. This was not feasible under our conditions. However, in experiments with young seedlings, the rate of infection was significantly higher (Table 5), indicating that method B should be the preferred choice as an inoculation technique. This is demonstrated by an experiment (Table 3) in which the rate was over 50% for a wide range of concentration for fast cc RNA-1. Under these conditions, the number of seedlings inoculated at each concentration (ten) is sufficient to detect a twofold difference in rate of infection.

The results reported here show that both circular and linear forms of fast cc RNA-1 are infectious, confirming results obtained with other viroids (1). There was no significant difference between infectivities of the fast cc RNA-1 and -2 forms but the fast form was significantly more infectious than the slow form. The results of the experiment in which the fast and slow cc RNAs were purified from the same palm show that the fast form with 246 nucleotides was more infectious than the slow form with 287 nucleotides (2). The extra repeated segment of 41 nucleotides appears to have a profound effect on infectivity: the addition of this duplicated sequence reduces the infectivity of the molecule from an average infection rate of 68% to less than 5%. Therefore, it is possible that the right-hand side of the native molecule plays a significant role in infectivity. The slow form is only found in infected palms at the time of appearance of the leaf symptoms (3); at earlier stages of disease only the fast form is present and this form is thought to be involved in natural spread of the disease (3). Therefore, the slow form appears to have a primary role in symptom development while the fast form may have its main role in infectivity and spreading of the disease.

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