Collection and Properties of Phloem Sap from Healthy and Lethal Yellowing-Diseased Coconut Palms in Jamaica

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ABSTRACT

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Techniques were devised for collecting phloem sap (coconut toddy) from inflorescences and cut trunks of coconut palms under field and laboratory conditions. Although inflorescence sap was obtained from cultivars showing both high and low susceptibility to lethal yellowing (LY) disease, it could not be obtained from the widely grown susceptible cultivar Jamaica Tall. Inflorescence sap had dry weights of 120–190 mg/ml and osmotic pressures (OPs) of 500–900 mosmol/kg; samples of trunk sap from healthy palms were more dilute (60–90 mg/ml), but had proportionately higher OPs (400–550 mosmol/kg). Volumes of trunk sap obtained from LY-diseased palms decreased as symptoms became more advanced, and the samples were more concentrated. Sap was not obtained from palms beyond the mid to late yellowing stages of the disease. No conclusive evidence was obtained for the presence or growth of mycoplasmalike organisms in sap from diseased palms, but samples from inflorescences and trunks of both susceptible and resistant cultivars were suitable as basal media for the growth of representative species of three genera of mycoplasmas.

The phloem environment of coconut palms (*Cocos nucifera* L.) is of special interest in research on lethal yellowing (LY) disease, which is thought to be caused by a phloem-limited mycoplasmalike organism (MLO) (1,17). This, and an accompanying report (19) describe the collection of phloem sap from coconut palms and the effects of LY disease on its production, physical properties, and chemical composition.

Large volumes of phloem sap can be obtained from certain groups of woody monocotyledons, notably within the Agavaceae and Palmae (20,21). In palms, this liquid is commonly known as toddy and coconut is one of several species that are commercially exploited for toddy production in various parts of the world (6). There is, however, no tradition of toddy tapping in the Caribbean region where cultivars have been limited to the Jamaica Tall (JT) type until comparatively recently (9), and the techniques described here were developed from those in published accounts (6,16) and from procedures developed by R. E. McCoy (*personal communication*). Because our ultimate interest was in the presence or growth of MLO in phloem sap, we also describe some observations on the comparative ultrastructural features of saps from diseased and healthy palms and report the suitability of phloem sap as a basal growth medium for three genera of mycoplasmas.

MATERIALS AND METHODS

Plant material. Most experiments utilized either cultivar JT, which is highly susceptible to LY, or cultivar Malayan Dwarf (MD), which shows very low susceptibility to the disease (2). Healthy MD palms and LY-diseased JT palms were sampled at various sites in Kingston and eastern Jamaica, where the disease is pandemic. Healthy JT palms were collected from a disease-free area at St. Ann's Bay, and exotic cultivars and hybrids were sampled from a cultivar trial at Fair Prospect, Portland.

Diseased palms were rated and selected on an LY severity scoring scale (7), similar to that described by McCoy (12), based on stages of nutfall and leaf yellowing. Nutfall stages: <10 nuts recently shed, score 1; >10 nuts shed, >10 nuts remaining on palm, score 2; <10 nuts remaining on palm, score 3. Yellowing stages: 20–40%, 41–60%, 61–80%, >80% of total leaves to L + 1 (see below) yellowed, necrotic, or recently shed, score 4–7, respectively; younger than L + 1 yellow but some leaves green, score 8; no green leaves, some yellow leaves remaining, score 9; and all leaves necrotic or recently shed, score 10. This scale was applied only to JT palms that had previously shown good growth and bearing.

Field collection from inflorescences. Coconut inflorescences are axillary and develop within a robust ensheathing spathe. Under favorable conditions, a mature inflorescence opens once a month and four or five younger spathes are visible in the crown of the palm. The most recently opened inflorescence provides a convenient reference point and is referred to here as I + 1; its subtending leaf (L + 1) can be readily identified from the ground. Toddy collections were attempted from unopened spathes in either the first or second younger position from the most recently opened inflorescence (designated I−1 and I−2).

Accounts of traditional methods of collecting coconut toddy refer to many exotic features (6), frequently including beating or bruising the inflorescence to induce or maintain the flow of sap. Various bruising methods were tried but a simpler pretreatment was finally adopted. The tip of the spathe was clipped and the sheath carefully peeled off. As the sheath was removed, the exposed inflorescence spikes were bound together firmly with paper masking tape until the inflorescence was enclosed in an artificial paper sheath. This enabled the inflorescence to be curved downwards to allow sap to drip off the cut end and facilitated slicing the exposed inflorescence spikes. Sap flow was initiated by cutting 5–10 cm off the tip of the bound inflorescence to expose the vascular tissues of the spikes, and was maintained by removing 3- to 5-mm slices from the cut end two or three times each day. To reduce fermentation, sap was collected in 50-ml tubes (the largest available) that were cooled in ice-packed vacuum flasks, clamped to the spathe (Fig. 1). The inflorescence stalk was sprayed with insecticide (Dichlorvos) and the whole assembly was enclosed in a polyethylene bag to exclude rain and flying insects. Sap was removed when the inflorescence was trimmed and stored frozen.

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Vacuum flasks retained ice for over 48 hr under field conditions, but collecting tubes often overflowed at night.

Laboratory collection from trunks. Palms were felled and the exposed fronds and inflorescences were removed to within the third or fourth oldest frond from the spear leaf. Sections of the crown (cabbage tissues), consisting of the lower 1 m of the remaining leaf bases and the top of the trunk to at least 20 cm below the growing point, were returned to the laboratory and stored overnight with the frond ends in water. Crowns were then either inverted in water or positioned horizontally with water percolating over the cut leaf bases. The trunks were then trimmed down to the soft immature tissues below the growing point and sap was pumped or allowed to drip off the cut surface and stored frozen. In most experiments, and for all samples collected for chemical analysis (19), a stream of moist nitrogen was introduced into a plastic bag sealed over the cut surface to reduce oxidation.

Osmotic pressure, pH, and dry weight determinations. Osmotic pressures (OPs) were determined on crude, unfiltered sap from freezing point depressions measured with a Beckmann thermometer. Measurement of pH was carried out on fresh or freshly thawed sap but readings were rapidly affected by exposure to air. Sap was clarified by filtration through glass fiber filters (Whatman GF/A) and dry weights were determined by lyophilizing 50 to 150 ml aliquots in preweighed flasks, followed by final drying to constant weight over phosphorus pentoxide. Filtration removed less than 0.2% of the dry weight.

Fig. 1. Field collection of toddy from an inflorescence of a Malayan Dwarf coconut palm. Collecting tube is cooled by crushed ice inside the vacuum flask.

Electron microscopy of sap. Up to 200-ml samples of unfiltered sap were sedimented by differential centrifugation for 15 min at 1,500 *g* followed by 30 min at 22,000 *g* (4 C), and the pellets were embedded in 2% (w/v) agar and fixed in 2.5% (w/v) glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). After postfixation in 1% (w/v) buffered osmium tetroxide the samples were dehydrated through an acetone series, embedded in Spurr's resin, sectioned, and examined with an AEI 801A electron microscope (22).

Culture of mycoplasmas in phloem sap. Stock cultures of *Acholeplasma laidlawii* (NCTC 10116) and *Mycoplasma fermentans* (NCTC 10117) were grown in conventional mycoplasma medium (10) and incubated at 30 or 37 C, respectively. *Spiroplasma citri* (NCPPB 2565) was grown in SMC medium (18) at 30 C. Samples of phloem sap were sterilized by passing through 0.45 μm filters (Millipore Corp.) and supplemented with combinations of heat-inactivated horse serum (20% v/v), fresh yeast extract (10) (10% v/v), serum fraction (Difco, 1% v/v) or used unsupplemented. Phenol red (20 μg/ml), penicillin G (300 IUs/ml) and thallous acetate (200 μg/ml) were included in all media, which were dispensed and incubated under air in airtight screw-capped vials or under sterile liquid paraffin in loosely capped test tubes. For quantitative growth experiments, phloem sap media were heated 30 min at 55 C, cooled, and inoculated with approximately 1% (v/v) of an actively growing subculture grown in a similar medium; this represented a maximum concentration of 0.01% (v/v) of conventional mycoplasma medium in the phloem sap test medium. Growth was monitored by acid production and light microscopy and compared by conventional plate counts or by streaking a loopful of the test medium onto conventional agar medium. Counts were carried out when a change in pH was first detected and after a further 24 hr.

RESULTS

Collection from inflorescences. Several early attempts to collect sap from inflorescences of JT palms met with little success, regardless of whether the inflorescences were bound, bruised, or sliced regularly and the palms were kept well watered. A maximum of 25 ml was collected over a 24-hr period on one occasion, but most palms, whether diseased or healthy, exuded only a few drops. It was subsequently found that sap could be collected readily from inflorescences of MD coconuts without bruising or other pretreatment, and a systematic examination of other palm cultivars indicated that the low yields of inflorescence sap were an unusual feature of the JT variety (Table 1). Unfortunately, no other Tall palm cultivars were sufficiently accessible for sap collection, but good yields of sap were obtained from Dwarf and intermediate cultivars showing both high (Rangiroa Dwarf) and low (Malayan Dwarf, Indian Dwarf) susceptibility to LY (2). Sap could not be obtained from a single JT palm in the early stages of LY disease development.

Collection from trunks. In contrast to inflorescences, cut trunks of JTs produced large volumes of toddy for several days. The mean yields of sap produced by eight healthy palm crowns over a 4-day period (horizontal position) are shown in Fig. 2A; data were calculated as rates of exudation over the preceding collecting periods as these were of unequal duration. Sap exudation reached a peak at 12–14 hr, when a maximum of 30 ml/hr was recorded from one crown, but declined to about 5 ml/hr within 1 wk. Comparable yields were obtained from palm crowns tapped vertically. When seven LY-diseased palms were tapped by the same techniques, yields decreased in approximate proportion to the stage of symptom development (Fig. 2B).

Properties of phloem sap. The dry weights and OPs of trunk sap samples from eight healthy and six diseased palms were

comparable and showed similar declines over the 4- to 5-day collection periods; however, both parameters tended to increase with advancing symptoms of LY (Fig. 3A and B). Sap from healthy MD trunks gave similar values. Comparison with data for sap collected from inflorescences of two MD palms growing in areas of high (Kildare, Portland) and low (Kingston) rainfall (Fig. 4) showed that although the total solute concentration of trunk sap was only about half that from inflorescences, the OP of the former was proportionately higher. Yields of inflorescence sap were not recorded as samples frequently overflowed the 50-ml collection tubes, but dry weights and OPs were closely correlated at both sites and increased in response to water stress at the drier region.

Freshly collected phloem sap from both sources was a clear or slightly opalescent sugary liquid varying from colorless to a pale straw color. Slight precipitation developed on autoclaving, concentrating in vacuo, dialysis against water and freezing. This accounted for less than 0.2% of the original dry weights in several samples tested and was removed from samples that had been frozen and thawed by coarse filtration (GF/A) or low-speed centrifugation prior to lyophilizing. Most freeze-dried samples were only slightly affected by oxidative browning when reconstituted. Fresh, chilled sap samples had pH values of 7.6–7.9. The pH was lowered by bacterial contamination but showed a marked increase on exposure to air. Samples that were deliberately aerated increased to pH 8.5–9.0 within 1 hr; this effect was not prevented by addition of reducing agents (sodium ascorbate, sodium thioglycollate or cysteine-HCl, all at 0.1% [w/v]) or phosphate buffer (to 0.1 M) but was reduced by autoclaving and did not occur in samples stored in sealed containers or under liquid paraffin.

Electron microscopy of sap. A striking feature of low-speed pellets of sap from both diseased and healthy palms was the presence of conglomerations of membranous material (Fig. 5A); this appeared to form the bulk of the precipitate described above that developed on freezing. High-speed pellets from both sources showed masses of vesicular bodies (Fig. 5B and C) and some of the structures seen in both diseased and healthy preparations showed a superficial resemblance to profiles of mycoplasmas. This made interpretation of micrographs difficult, but no consistent features

TABLE 1. Volumes of toddy collected from single inflorescences of healthy (H) and lethal yellowing (LY)-diseased Jamaica Tall coconut palms and six exotic cultivars at Fair Prospect, Portland

Variety	Susceptibility to LY[a]	Volumes (ml) per day from start of collection 1	2	3	4	5	6	Totals
Jamaica Tall (H)	+++	2	5	6	5	4	4	26
Jamaica Tall (LY)		0	0	0	0	0	0	0
Fiji Dwarf	++	40	95	137	158	126	94	650
Indian Dwarf	+	61	180	325	358	439	309	1,672
King Coconut	+	25	74	120	96	146	53	514
Malayan Dwarf	+	87	130	191	271	289	126	1,094
Rangiroa Dwarf	+++	62	98	135	157	249	171	872
Spicata	n.d.	63	76	92	103	120	109	563

[a] Scored as: + = low susceptibility (<10% lost in field trials); ++ = intermediate susceptibility (10–90% lost in field trials); +++ = high susceptibility (>90% lost in field trials); and n.d. = not determined.

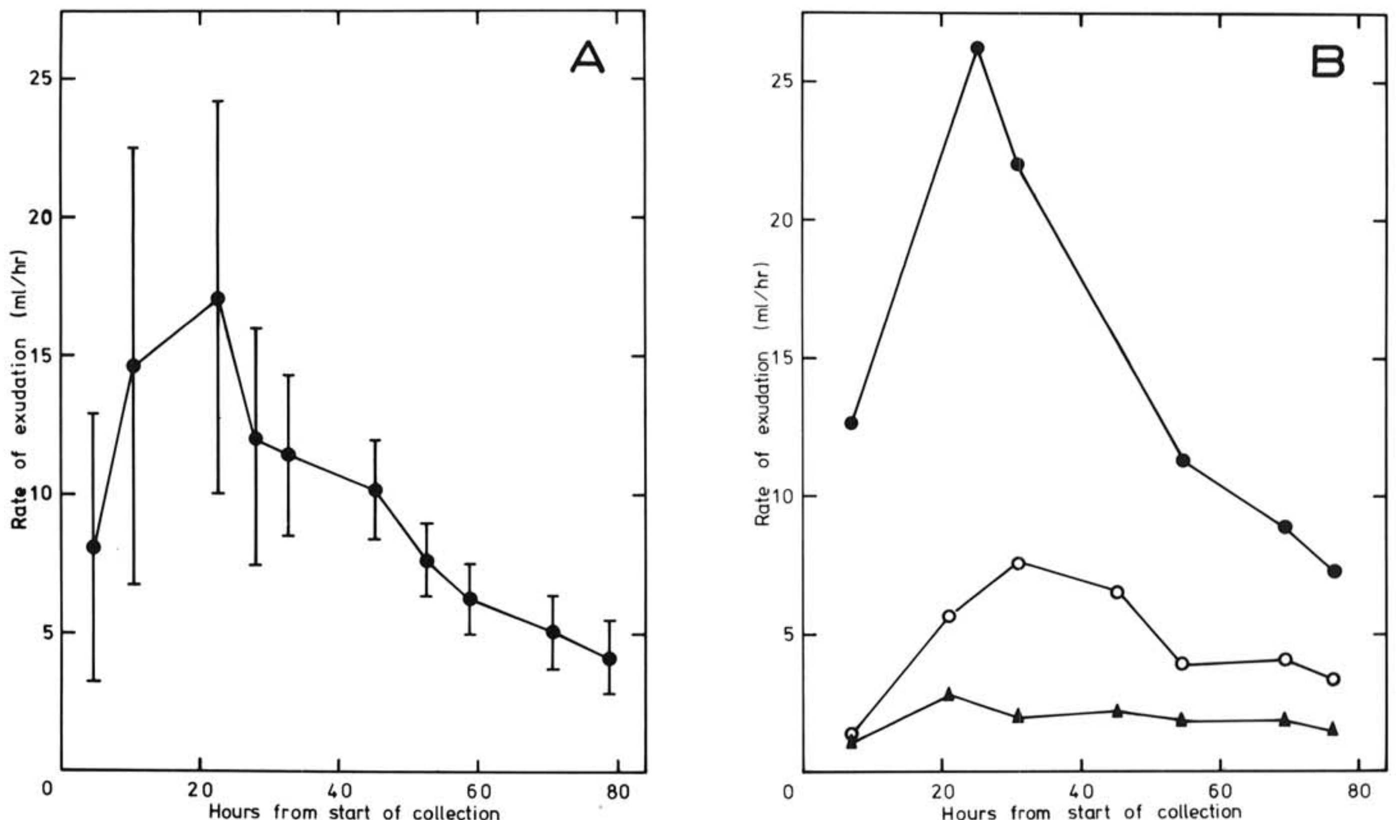
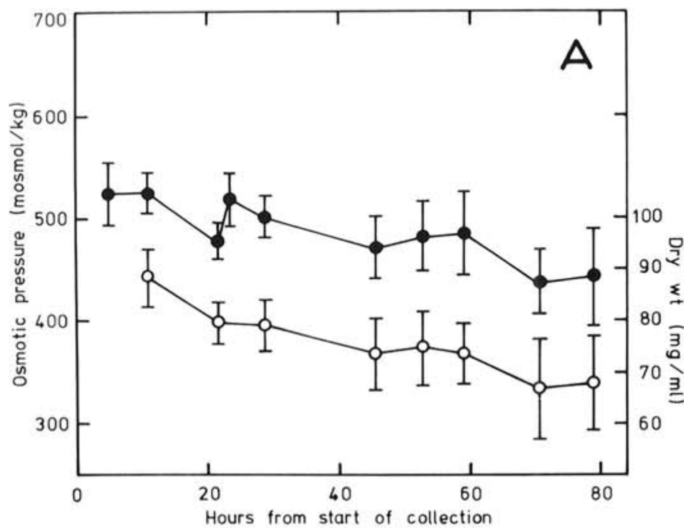


Fig. 2. Production of phloem sap from cut trunks of Jamaica Tall coconut palms in the laboratory. Each point shows the rate of exudation (ml/hr) over the preceding time interval. **A,** Mean ± 1 standard deviation for eight healthy palms; **B,** lethal yellowing disease stage 2 (●—●), 1 palm; stages 4–5 (o—o), mean of three palms; stages 6–7 (▲—▲), mean of three palms.



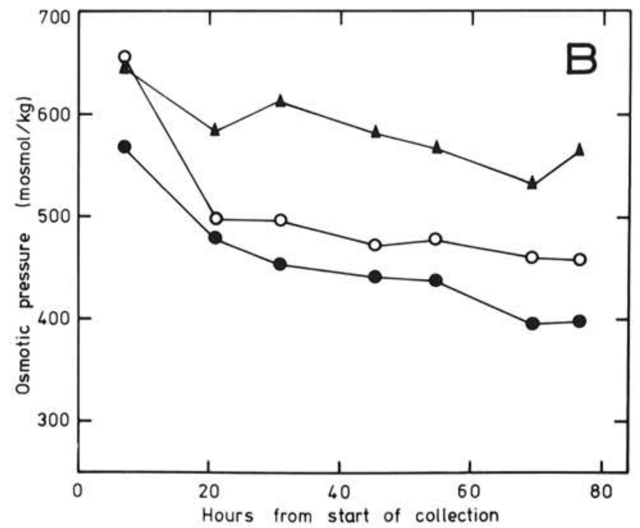


Fig. 3. Osmotic pressures and dry weight contents of phloem sap from cut trunks of Jamaica Tall palms. Each point relates to sap collected over the preceding time interval. **A,** Mean ± 1 standard deviation osmotic pressures (●—●) and dry weights (o—o) for eight healthy palms; **B,** lethal yellowing disease stage 2 (●—●), one palm; stages 4–5 (o—o), mean of three palms; stages 6–7 (▲—▲), mean of two palms.

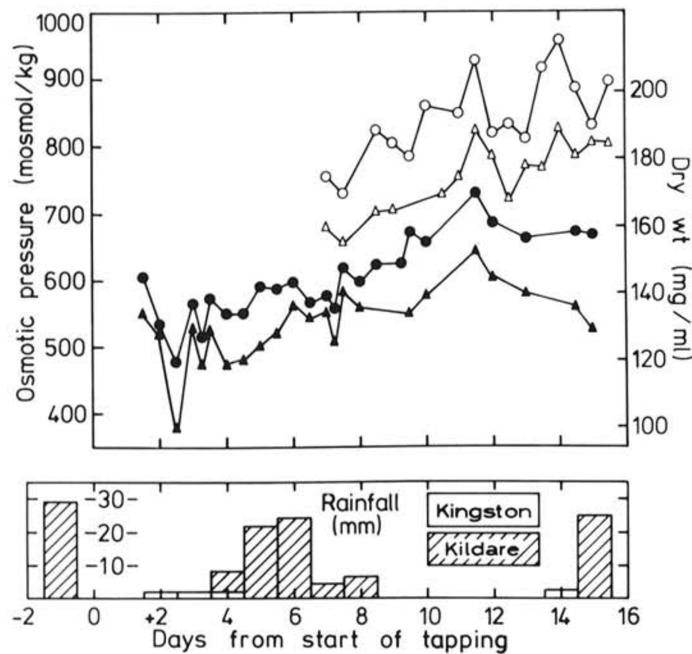


Fig. 4. Osmotic pressures (circles) and dry weight contents (triangles) of phloem saps collected in the field from single inflorescences of Malayan Dwarf palms growing in areas of high rainfall in Jamaica (Kildare, closed symbols) and low rainfall (Kingston, open symbols). Yields of sap at the Kingston site were initially very low and data were not obtained for the first seven days after preparing the inflorescence for tapping.

suggesting the presence of MLO in sap from diseased palms were detected in samples examined from 12 diseased and seven healthy palms.

Growth of mycoplasmas in phloem sap. Samples of 0.45 μm of filtered sap from trunks of both diseased and healthy palms incubated for 2–3 days at 30 C under liquid paraffin frequently showed a drop in pH from about 7.4 to 6.8, associated with anaerobiosis towards the bottom of the culture tubes. This effect was not prevented by the addition of 1 mg/ml tetracycline-HCl or 10 mg/ml thimerosal (both concentrations inhibitory to *A. laidlawii*) but was eliminated by heating 30 min at 55 C. Although low concentrations of submicron debris and vesicular bodies could sometimes be detected, light microscopic observations on sap from both diseased and healthy palms showed no consistent differences and could not be interpreted to reflect the growth of LY MLO.

TABLE 2. Growth of mycoplasmas in phloem sap and conventional media

Organism	Medium[a]	Log₂ increase at[b] t	t + 24
Acholeplasma laidlawii	Sap	11.12	11.65
	Sap + SF	12.91	13.28
	M	9.40	13.35
	M + SF	16.61	17.06
Mycoplasma fermentans	Sap + SF	2.56	2.46
	Sap + SF + YE	11.19	12.91
	M + S	12.09	15.37
Spiroplasma citri	Sap	trace	trace
	Sap + SF	9.10	10.06
	SMC + S	9.86	11.75

[a] Filter sterilized (0.22 μm) phloem sap, conventional mycoplasma (M) or spiroplasma (SMC) medium supplemented with 1% serum fraction (SF), 10% yeast extract (YE) or 20% horse serum (S).

[b] Determined from colony counts on conventional media by subtracting $\log_2$ of the counts per milliliter at time of inoculation from $\log_2$ of the counts per milliliter when a change in pH was first detected (t) and after a further 24 hr (t + 24).

Apart from three isolates of *A. axanthum* (8), no mycoplasmas were isolated from uninoculated phloem sap.

Preliminary experiments showed that *A. laidlawii* grew well in unsupplemented, filter-sterilized sap collected from diseased and healthy palm trunks and produced small 'fried egg' colonies when sap was solidified with 1% (w/v) agar and incubated under nitrogen. Growth of strains of *A. axanthum* in similar samples of phloem sap led to the discovery of further *Acholeplasma* isolates from palms (8). Unsupplemented sap from the same sources sometimes supported moderate growth of *S. citri* but this organism grew consistently well when 1% (w/v) serum fraction was included and produced small colonies on serum fraction-supplemented sap solidified with 1.2% (w/v) agar. Good growth of *M. fermantans* was obtained in sap supplemented with 1% (v/v) serum fraction and 10% (v/v) fresh yeast extract. The increases in cell numbers of all three organisms in sap containing these supplements were comparable to those obtained in conventional media (Table 2). Growth of the three mycoplasmas in inflorescence sap from MD palms was not quantified but three samples of sap (each 10 ml) containing the same supplements were inoculated with 2 μl of late exponential growth phase cultures grown in conventional medium and incubated under similar conditions. Acid production, light microscopy, and plating on conventional media showed that *A. laidlawii* grew well in all three unsupplemented sap samples, *S. citri* grew in one sample of unsupplemented sap and in all three samples

supplemented with 1% (v/v) serum fraction, and *M. fermantans* grew in one sample supplemented with either 1% (v/v) serum fraction or with 1% (v/v) serum fraction + 10% (v/v) fresh yeast extract. These results confirm the earlier observations by McCoy (13,14).

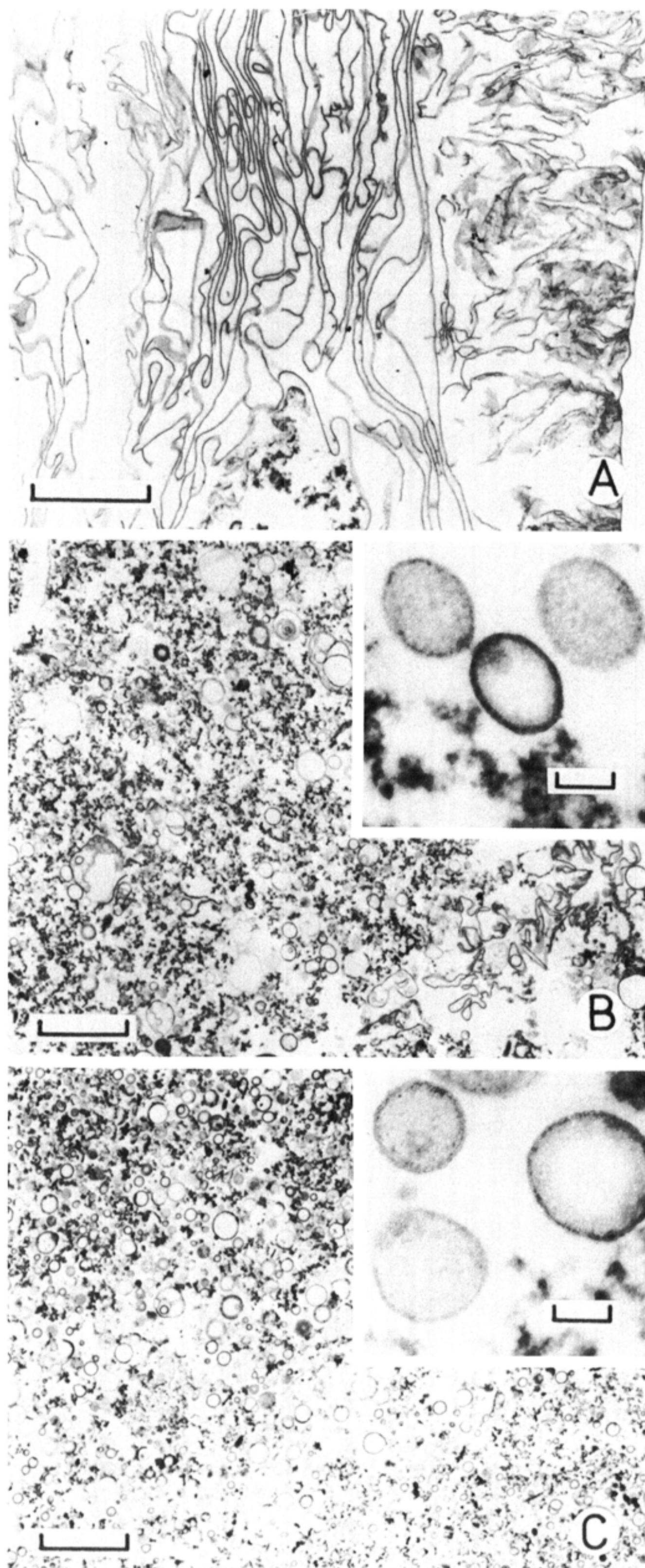


Fig. 5. Electron micrographs of ultrathin sections of pellets after differential centrifugation of phloem saps from cut trunks of Jamaica Tall palms. **A,** Sap from a lethal yellowing-diseased palm after low-speed centrifugation (1,500 *g*), showing conglomerations of membrane profiles (bar represents 2 μm); healthy sap (not shown) gave similar preparations. **B and C,** preparations from diseased and healthy saps, respectively, after high-speed centrifugation (22,000 *g*), showing abundant vesicular bodies in saps from both sources. Insets show resemblance of some vesicles to profiles of mycoplasmalike organisms at higher magnification. Bars represent 2.0 and 0.1 μm.

DISCUSSION

Although the yields of sap that we obtained from coconut palm inflorescences were somewhat lower than reported in contemporary sources (6,11), most of the cultivars that we were able to tap were dwarf or semidwarf types that are known to give lower yields than the Tall palm cultivars that are exploited for toddy commercially. Maximal yields may also require more prolonged tapping and irrigation of the palm. These factors were unlikely to explain the lack of sap production from JT inflorescences, however, which appeared to be an unusual feature of this cultivar. This may explain the absence of toddy tapping in the Caribbean, where immigrant groups, notably from the East Indies, must have been familiar with the techniques and their application.

Exudation of sap from cut coconut crowns provided a more convenient technique for collecting sap from all the cultivars in the laboratory, where exudation and microbial contamination could be controlled more easily. Sap collected from trunks showed a consistently lower concentration of total solids than that from inflorescences, but had a proportionately higher OP. This probably reflected the higher concentration of inorganic solutes in trunk sap, but there were otherwise few qualitative differences between saps from the two sources (19). The most obvious effect of LY was to reduce the volume of sap produced, as palms with more than half the canopy affected by yellowing produced little or no sap. The concentration of total solids also tended to increase as the disease progressed. These findings are consistent with a disturbance in phloem function in diseased palms, but provide no evidence as to its cause. Our electron microscopic observations on the phloem of diseased palms (*unpublished*) suggest that the highest concentrations of MLO occur at sink sites, such as expanding leaf base tissues, inflorescences and root tips, and mechanical blockage of phloem by MLO or callose seems unlikely to account for the observed reduction in exudation. Similar conclusions have been drawn in studies of translocation in mycoplasmal diseases of other woody perennials (3,4). Other authors (15) have detected a reduction in xylem tension in diseased palms which, from considerations of mass flow, would favor an increase in phloem water potential.

The growth of mycoplasmas in phloem sap indicates that the physicochemical environment within phloem sieve elements is favorable for the growth of these organisms. These observations require cautious interpretation, however, as they provide no direct evidence that the MLO present in sieve elements are, in fact, related to the class Mollicutes. We were not able to detect the unequivocal presence or growth of LY MLO in toddy collected from diseased palms, but it would be useful to include phloem sap in studies of the survival and multiplication in vitro of MLO associated with other yellows diseases where presence of the pathogen can be monitored by a sensitive infectivity bioassay system (5).

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