Flagellate Protozoon Associated with Poor Development of the Root System of Cassava in the Espirito Santo State, Brazil

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ABSTRACT

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"Chochamento das raizes" (empty roots) is a problem of increasing importance in cassava (*Manihot esculenta*) culture in the northern Espirito Santo state, Brazil. It affects particularly the cultivar Unha, the most cultivated in this region, and is characterized by poor development of the root system and general chlorosis of the aerial parts. A flagellate protozoon (*Phytomonas* sp.) was found associated with the diseased plants, living in the lacticifer ducts. The protozoon is easily detected by light microscopy in the smears of latex exuded from cut wounds. Under the electron microscope, the flagellates show the typical subpellicular layer of microtubules, single flagellum with 9+2 axial complex and paraxial structure, basal body, and kinetoplast. Field surveys showed that about 50% of the chlorotic Unha cassava plants contained the flagellate in the latex, whereas none was found in symptomless plants. Under greenhouse conditions, the flagellate was transmitted only by grafting. Attempts to transmit the protozoon by injecting or pricking latex suspensions into healthy plants or using a Tingidae hemiptera (*Vastiga* sp.) as vector have been unsuccessful.

Additional key words: electron microscopy, lacticifer.

Espirito Santo is not among the largest cassava (*Manihot esculenta* Crantz.)-producing states in Brazil. Nevertheless, cassava culture is one of the most important in its northern region, mainly for flour (starch) production. A disease characterized by poor development of the root system, resulting in small and slender roots, a lack of starch, and general chlorosis of the aerial parts has been noticed since 1979, affecting in particular the cultivar Unha, which is the most commonly planted. There are some indications that the disease is spread by an unknown aerial vector. After the appearance of few initially diseased plants, the condition might spread randomly throughout the plantation, attacking 50% or more of the plants and resulting in heavy losses (17). Initial studies did not demonstrate fungi, bacteria, insect toxin, soil problems, or nematodes associated with this disease, known locally as "chochamento das raizes," which means "empty roots." Possible viral or mycoplasmal etiology was considered, and samples were sent from Empresa Capixaba de Pesquisa Agropecuaria (EMCAPA), where the initial studies (17) were made, to the Universidade de Brasilia, for electron microscopic and transmission studies. Subsequent investigations indicated that a flagellate protozoon was associated with the disease. This paper gives the details of this finding.

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MATERIALS AND METHODS

Initial samples received from EMCAPA consisted of leaves and stems from healthy and diseased Unha cassava plants. Later, cuttings from both healthy and affected plants were sent; they were planted in 10-L plastic bags filled with soil and left to root under greenhouse conditions.

Light microscopy. Smears of the latex exuding from the cut wounds of stem, petiole, and root tissues were fixed with methanol for 5 min and then stained for approximately 20 min with 10% Giemsa in 0.02 M neutral phosphate buffer (18). Fresh latex suspensions also were examined without staining by phase-contrast microscopy.

Transmission electron microscopy. Small tissue pieces from leaf petioles and stems were fixed in 2% glutaraldehyde and 2% paraformaldehyde solution in 0.05 M, pH 7.2 sodium cacodylate buffer under light vacuum for 2–3 hr, postfixed in 1% OsO_4 in the same buffer for 1 hr, and left overnight in 0.5% uranyl acetate for

block staining. Fixed tissues were embedded in Spurr's low-viscosity medium and sectioned in an LKB Ultratome III microtome equipped with a Du Pont diamond knife. Sections were stained with 2% uranyl acetate and Reynolds' lead citrate and examined in a JEOL-JEM 100C electron microscope.

Scanning electron microscopy. Longitudinal or transverse sections of leaves and petioles of healthy and diseased Unha cassava plants were fixed in the same fixative used for transmission electron microscopy. After 2 hr of fixation, the tissues were dehydrated in an ethanol series, dried at critical point, and coated with gold in a sputter-coater. Also, flagellate-containing latex suspensions were fixed in the same fixative, to which 0.9% NaCl was added. After 2 hr of fixation, the suspension was transferred onto 0.1% polylysine-coated coverslips, left for 20 min, and then processed for scanning electron microscopy (SEM) as described above for tissue fragments.

Transmission tests. Attempts to transmit the flagellate from diseased plants to healthy ones were made by fork-grafting or budding affected material on top of the healthy plants or by approach-grafting a flagellate-invaded plant with a healthy one. Mechanical inoculation with flagellate-containing latex suspensions was made by injecting suspensions or by pricking with a brush made of cotton wool and capillary tubes onto leaves, petioles, and stems of healthy cassava plants. Insect transmission was tested with the Tingidae hemiptera (*Vastiga* sp.), commonly present in cassava plantations. The insects were collected from the field, reared on diseased plants for about 1 wk, and then transferred to healthy Unha plants and kept for 2–3 wk. Transmission in these experiments was periodically evaluated by examination of latex smears from inoculated plants.

RESULTS

Initial evidence that a flagellate protozoon was involved with diseased plants came from the examination of thin sections of leaves and petioles. Membrane-bounded bodies of various shapes and sizes were observed consistently in the vessels of the lacticifer (Figs. 1–3). A more careful examination of such bodies revealed in each of them a subpellicular layer of microtubules and, depending on the sectioning plane, kinetoplast, basal body, nucleus, and other cell structures. In favorable longitudinal sections, flagella could be observed emerging from flagellar pockets and also near the cell bodies (Figs. 3 and 4). The flagella exhibited the typical 9+2 axial complex and the paraxial rod, with fine periodicities (Fig. 3, inset; Fig. 5). In a few dividing cells, an intranuclear array of microtubules was discernible, indicating an endomitotic process (Fig. 2). These flagellates could not be found in other cell types or in healthy plants. Viruslike or cytopathic effects attributable to viral infection, as well as mycoplasma or rickettsialike structures in the phloem or xylem vessels, were not observed in the diseased tissues. The distribution of the flagellate cells was, however, irregular, and not every lacticifer duct in diseased plants contained them.

These flagellates were found in large numbers in the smears of latex exuded from cut wounds from several parts of the diseased plants. The flagellate cells were slender and fusiform and had a single flagellum. Kinetoplasts were apparent at the base of the flagellum in Giemsa-stained preparations (Fig. 6). Some latex samples collected from different parts of the same plant occasionally failed to yield flagellates, while in others the organisms were easily observed.

Field surveys carried out at Linhares, ES, and surrounding counties revealed that roughly half of the 80 sampled chlorotic plants contained the flagellates in their latex, while none of the symptomless plants examined was affected. Another survey made at the cassava germ plasm collection at EMCAPA, Vitoria, ES, showed that of 625 sampled plants (five of each of the 125 lines and varieties), only four had flagellates in their latex. Similar work was carried out at Centro de Pesquisa Agropecuaria do Cerrado at Planaltina, DF. None of nearly 100 lines and cultivars of this collection (sampling of three plants per line) contained the flagellates. Also, in 13 cultivars received from the Centro Nacional de Pesquisas da Mandioca e Fruteiras, Cruz das Almas, BA, protozoa could not be found in the latex.

Transmission tests were performed under greenhouse conditions by using plants derived from affected cultivar Unha cuttings as the source of inoculum. Flagellates were detected in about 33% of the cuttings that rooted, and they were used for further work. Fork-grafting of stems from affected plants on tops of healthy plants resulted in 70% (7/10) take, and flagellates were detected in all of them 3–4 wk later. All six bud unions survived, but the flagellates were found in only four of the budded plants. Of the five approach-grafts made, three took and in all of them the flagellate was transmitted from affected to healthy plants. Plants inoculated either by injecting or pricking with cotton wool dipped in the flagellate-containing latex suspensions (five for each treatment) failed to become infected. Of the five plants subjected to *Vastiga* sp. (10 individuals per plant), previously reared for 1 wk on diseased plants, none became infected.

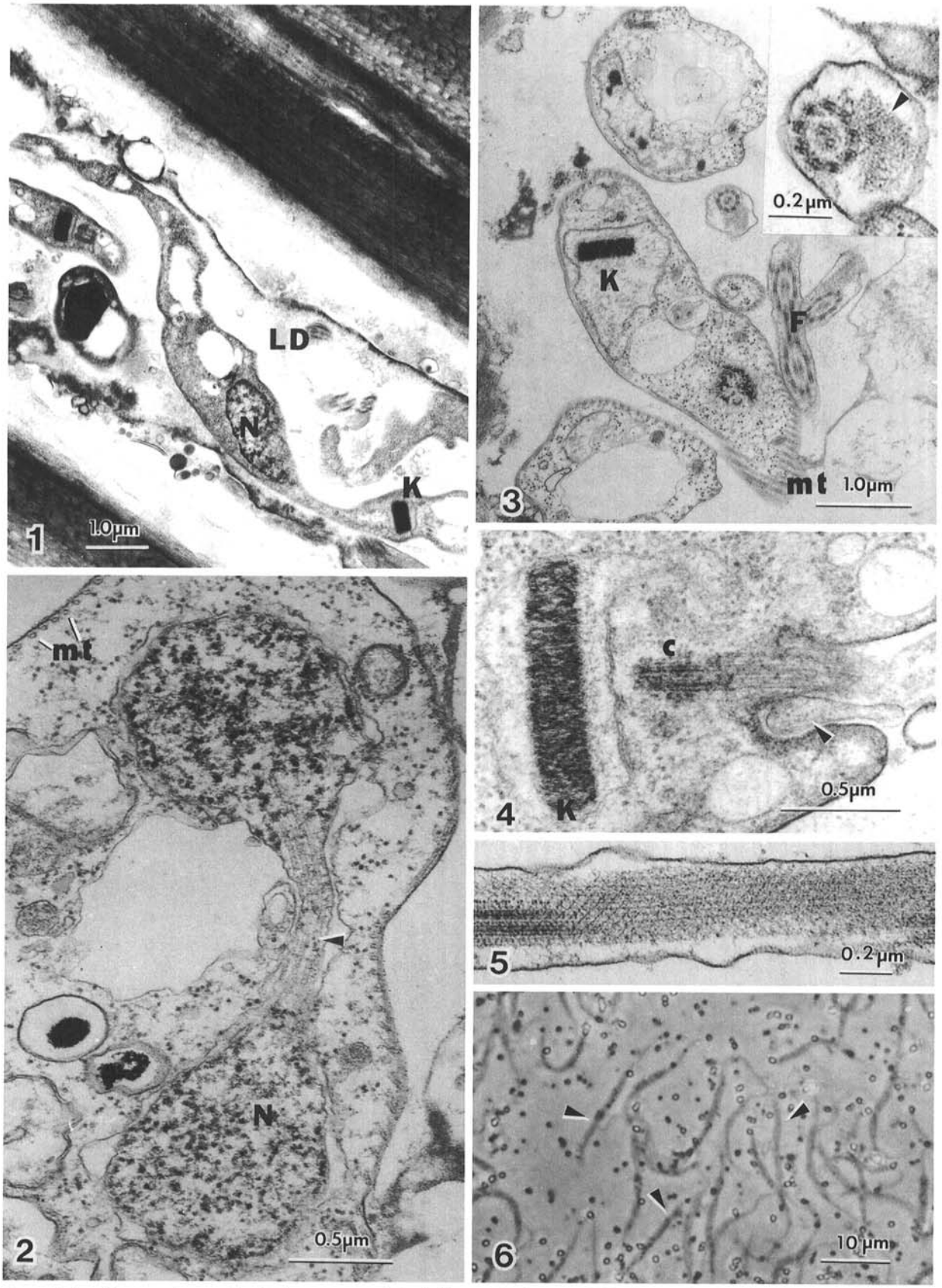
Electron microscopy of four of the plants infected by stem grafting revealed flagellates present in their lacticifers. Lacticifer ducts were easily identified because of their content and position. They have few recognizable cell structures and are usually rich in granules and amorphous masses. They occur near the vascular bundles in the leaves, while in the petioles they appear close to the collenchyma layer, a few cells below the epidermis and adjacent to the vascular bundles. The flagellates' cells could be seen individually or in small aggregates interspersed with the lacticifer content. It was difficult to assess the three-dimensional configuration of the flagellates based only on thin sections, but this was easily recognizable by SEM, either of latex suspensions (Fig. 7) or tissue fragments from leaves or petioles (Figs. 8–10). The flagellate protozoons (as already shown by light microscopy) were elongated, fusiform with a single flagellum (5.5 to 12 μm long) in one of the cell ends, and the whole cell was helically twisted (Figs. 7, 9, and 10). These cells were 0.5 to 1.1 μm wide and 8.2 to 18 μm long. Average distance from nucleus to kinetoplast was 3 μm. In the SEM of tissue fragments, flagellates commonly appeared mixed with an amorphous mass of dried latex that appeared to be plugging the lacticifer (Fig. 8). Occasionally they formed large aggregates free from the latex (Fig. 9). In such aggregates, the flagellates usually formed a "bouquet" type of arrangement, joined by their flagella.

DISCUSSION

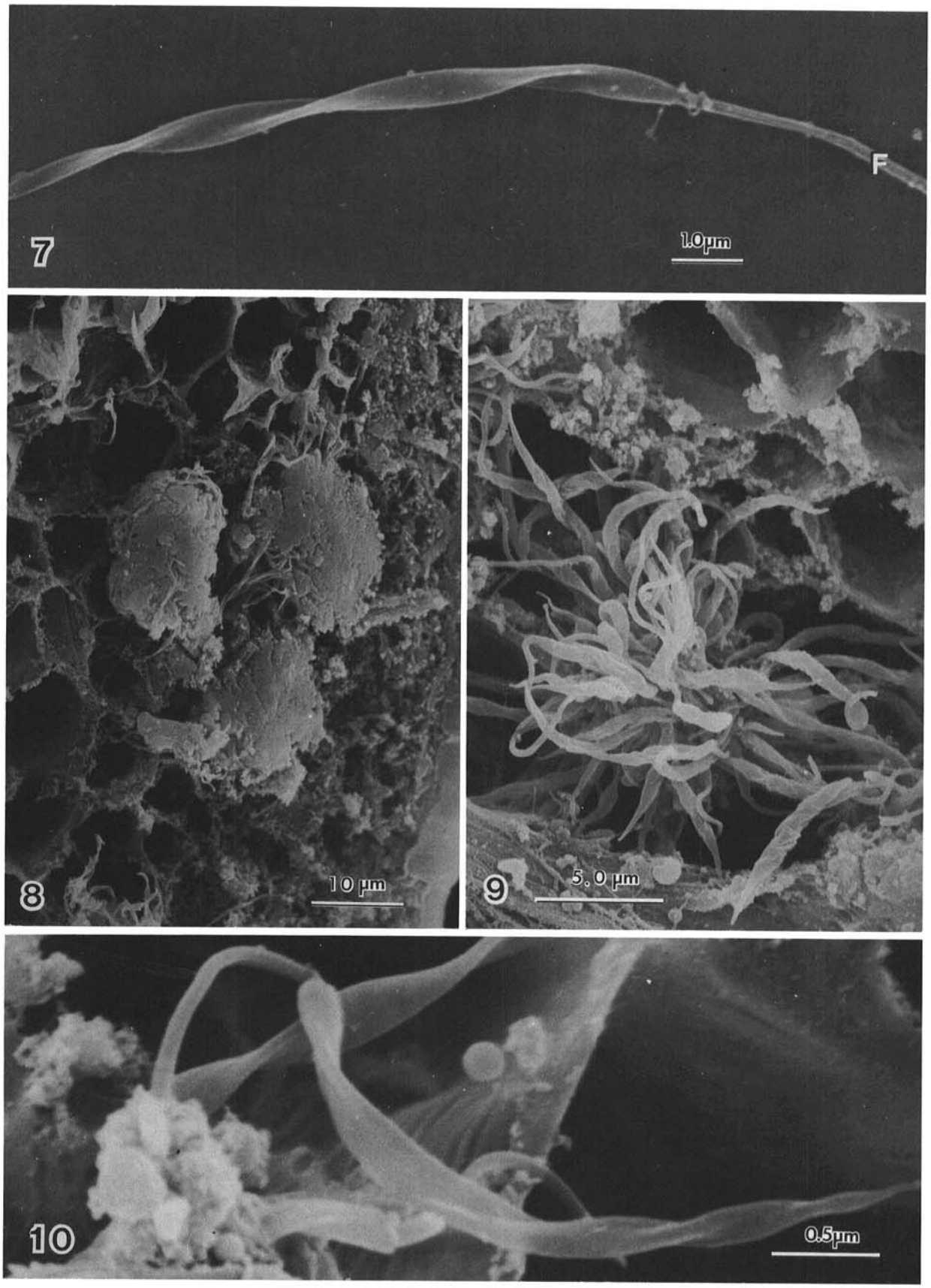
Trypanosomatid flagellates in plants have been known since 1909 when Lafont (12) reported them in three *Euphorbia* species. Flagellates have been repeatedly found in lacticifers of several Euphorbiaceae and Asclepidaceae species. Their taxonomic status is still rather confusing. *Phytomonas*, the genus originally suggested by Donovan (9) is most widely accepted by the protozoologists as plant flagellates. Such classification problems stem from difficulties in cultivating these organisms in vitro for careful comparative studies. Only one species, *Phytomonas davidi* Lafont, isolated by McGhee and Postell (13) from three *Euphorbia* species, was deposited in the ATCC (30.287). Recently, another *Phytomonas*, found in *E. pinea* L. and *E. characias* L., was successfully grown axenically in a complex medium (6,7).

The flagellate found in diseased cassava has the basic morphological characteristics of those previously described in several plant species and will thus be referred to as a *Phytomonas* sp. The species designation will await further comparative work with other known species. The peculiar helical cell, barely visible in the light micrographs but clearly depicted in the SEM images, is a distinguishing morphological feature. Similar helical cells were seen in *Phytomonas* sp. found in sieve tubes of "hartrot"-affected coconut (*Cocos nucifera* L.) plants (3) and in the lacticifer of *E. hyssopifolia* L. (4). Preliminary attempts to grow the *Phytomonas* sp. from cassava axenically have been successful in a biphasic medium containing blood in the solid phase (20), as was done for *P. davidi* (13). More details will be presented elsewhere (21).

Whether or not *Phytomonas* sp. is pathogenic to invaded plants is still an open question in the case of symptomless hosts. However, there are at least three well-documented cases of plant diseases associated with *Phytomonas* sp.: the phloem necrosis of *Coffea liberica* Bull. ex. Hiern. in Surinam (19), "marchitez sorpressiva" of



Figs. 1–6. Transmission electron micrographs of *Phytomonas* sp. in lacticifer vessels of cassava (*Manihot esculenta*) affected by "chochamento das raizes" disease. **1,** Longitudinal section through the flagellate within a narrow lacticifer duct (LD). K = kinetoplast and N = nucleus. **2,** A dividing cell of *Phytomonas* sp. Remnants of spindle tubules (arrowhead) are still visible within the dividing nucleus (N). mt = subpellicular microtubule. **3,** Group of cells of *Phytomonas* sp. in a large lacticifer lumen. Inset shows a detail of the cross section through the flagellum. Arrowhead points to the paraxial body at the side of the 9+2 axial complex. F = flagellum, K = kinetoplast, and mt = subpellicular microtubule. **4,** Longitudinal section at the base of the flagellum which emerges from the flagellar pocket (arrowhead). c = centriole and K = kinetoplast. **5,** Longitudinal section of the paraxial body within the flagellum. Note the fine periodicities. **6,** Phase-contrast light micrograph of a latex smear of diseased plant, stained with Giemsa. A large number of flagellates (arrowheads) are easily discernible.



Figs. 7–10. Scanning electron micrographs of the *Phytomonas* sp. associated with the flagellate disease of cassava. **7,** Cell of *Phytomonas* sp. in a latex suspension. Note the flagellum (F) emerging from one end of the helically twisted cell body. **8,** Low magnification image of the surface of a sectioned petiole. Masses of latex, intermixed with flagellate cells, appear plugging the lacticifer ducts. **9,** A detail of a group of cells of *Phytomonas* sp. forming a "bouquet" configuration. **10,** A higher magnification of a single cell of *Phytomonas* sp. emerging from the latex mass.

the oil palm (*Elaies guineensis* Jacq.) (8), and the "hartrot" of coconut palm (16). Both of these palm diseases are restricted to South America. A recent outbreak in the southern Bahia state of Brazil has been reported (5,15). In none of these cases, however, was the agent isolated, and Koch's postulates could not be completed.

Not all reports of successful mechanical transmission of *Phytomonas* sp. (10,14) have been confirmed. Our attempts have also been unsuccessful, possibly because internal pressure of the lacticifer ducts is very high. Thus, it is difficult for the flagellates in the inoculum to invade them.

There have been several positive cases of insect transmission of *Phytomonas* sp. After the first description by França (11) of the role of *Stenocephalus agilis* Scop. in the transmission of *Leptomonas davidi* Lafont to *E. plepeus* L., there have been several other reports. These involved Lygaeidae or Coreidae families of hemiptera (6). We could not find insects of these taxonomic groups in cassava plantings. The only hemiptera tested was *Vastiga* sp. (Tingidae), commonly infesting cassava plantations, which did not transmit the *Phytomonas* sp. from cassava.

Knowledge of cassava infection by *Phytomonas* sp. is not new. Aragão described *Phytomonas françai* Aragão in the latex of *M. palmata* (Vellozo) Pax. in 1927 but could not associate the invasion by the flagellate with disease (1,2). He managed to keep *P. françai* alive for several passages in a Noeller medium, apparently without cell multiplication. He succeeded, as we did, in transmitting the flagellate by approach-grafting. Besides the *Phytomonas* sp. from cassava, only *P. leptovassorum* Stahel associated with coffee phloem necrosis was transmitted by tissue union (19,22).

Although more information, especially the completion of Koch's postulates, is required to demonstrate that *Phytomonas* sp. is responsible for the disease of cassava, the constant association between its presence in the lacticifer and the disease and the absence of other pathogens are favorable evidence for such a view. The fact that not all plants in the field with chlorotic symptoms harbor the *Phytomonas* sp. might be explained by their irregular distribution within the invaded plants. It is likely that the temperature might affect such a distribution, since preliminary data have shown that higher temperatures inhibit the cell growth in axenic cultures (20). Furthermore, chlorotic symptoms of the aerial parts are probably not primary and might have causes other than infection by *Phytomonas* sp. The fact that the cultivar Unha is the most affected in a region where the disease appears to be endemic suggests that it is more susceptible to the *Phytomonas* sp. than other cultivars. In greenhouse conditions, however, preliminary tests have shown that several other cassava lines and cultivars are easily infected with *Phytomonas* sp. by grafting.

The main difference of the cassava disease-associated *Phytomonas* from other pathogenic flagellates is that it invades only the lacticifer, whereas those found in coffee and palm trees inhabit the phloem. The diseases caused by the latter probably result from interference with phloem transport and/or production of toxinlike substances. The poor root development in the diseased cassava could well arise either from imbalance in the metabolism of hydrocarbons, brought about by the invasion of *Phytomonas* sp. of the lacticifer ducts or in production of toxinlike metabolites.

LITERATURE CITED

1. Aragão, H. B. 1927. Sur un flagelé du latex de *Manihot palmata*: *Phytomonas françai* n.sp. C. R. Soc. Biologie 97:27.
2. Aragão, H. B. 1929. Pesquisas sobre *Phytomonas françai*. Mem. Inst. Oswaldo Cruz 25:299-304.
3. Attias, M., Bezerra, J. L., Oliveira, D. P., Costa, C. G., Machado, R. D., and De Souza, W. 1985. Detecção de *Phytomonas* em *Cocos nucifera* no Estado da Bahia. Estudo ultraestructural. Resumos XIa. Reunião Anual Pesquisa Básica em Doença de Chagas (Caxambú) BI-29. 39 pp.
4. Attias, M., and De Souza, W. 1985. Isolamento e cultivo de *Phytomonas* sp. do latex de *Euphorbia hyssopifolia*. Resumos XIa. Reunião Anual Pesquisa Básica em Doença de Chagas (Caxambú) BI-30. 39 pp.
5. Bezerra, J. L., and Figueiredo, J. M. 1982. Ocorrência de *Phytomonas staheli* Mc Ghee & Mc Ghee em coqueiro (*Cocos nucifera* L.) no Estado da Bahia, Brasil. Fitopatol. Bras. 7:139-143.
6. Dollet, M. 1984. Plant diseases caused by flagellate protozoa (*Phytomonas*). Annu. Rev. Phytopathol. 22:115-132.
7. Dollet, M., Cambrony, D., and Gorgani, D. 1982. Culture axenique in vitro de *Phytomonas* sp. (Trypanosomatidae) d'*Euphorbe* transmis par *Stenocephalus agilis* Scop. (Coreidae). C. R. Acad. Sci., Ser. III, Paris 295:547-550.
8. Dollet, M., Gianotti, J., and Ollangnier, M. 1977. Observation de protozoaires flagellés dans les tubes cribles de Palmiers a huile malades. C. R. Acad. Sci., Paris 284:643-645.
9. Donovan, C. 1909. Kala-azar in Madras, especially with regard to its connection with the dog and bug (*Conorrhincus*). Lancet 177:1495-1496.
10. França, C. 1914. La flagellose des Euphorbes. I. Arch. Protisenk. 34:108-132.
11. França, C. 1919. L'insect transmitteur de *Leptomonas davidi*. Bull. Soc. Pathol. Expt. 12:513-514.
12. Lafont, A. 1909. Sur la présence d'un parasite de la classe Flagellés dans le latex de *Euphorbia pilulifera*. C. R. Soc. Biologie 66:1011-1013.
13. McGhee, R. B., and Postell, F. J. 1946. Axenic cultivation of *Phytomonas davidi* Lafont (Trypanosomatidae), a symbiote of lacticiferous plants (Euphorbiaceae). J. Protozool. 23:238-241.
14. Noc, F., and Steven, L. 1911. Presénce a la Martinique de "*Leptomonas davidi*" Lafont. Bull. Soc. Pathol. Expt. 4:461-464.
15. Oliveira, D. P., and Bezerra, J. L. 1982. Ocorrência de "marchitez sorpressiva" do dendezeiro no Estado da Bahia, Brasil. Theobroma 12:107-108.
16. Parthasarathy, M. V., van Slobbe, W. G., and Soudant, C. 1976. Trypanosomatid flagellate in the phloem of diseased coconut palms. Science 192:1346-1348.
17. Silveira, J. S. M., and Pereira, A. V. 1983. Anomalias de causa desconhecida em mandioca (*Manihot esculenta* Crantz.) no estado de Espirito Santo. Anais Terceiro Congresso Brasileiro de Mandioca (Brasilia, DF). 73 pp.
18. Simpson, L., and Braly, P. 1970. Synchronization of *Leishmania tarentolae* by hydroxiurea. J. Protozool. 17:511-517.
19. Stahel, G. 1931. Zur Kenntinis der Sieberöhrenkrankheiten (Phlöemnekrose) des Kafeebaumes in Surinam. I. Mikroskopische Untersuchungen und Infektionversuche. Phytopathol. Z. 4:65-82.
20. Vainstein, M. H. 1984. Um *Phytomonas* associado ao "chochamento" das raizes da mandioca. M.S. dissertation, Univ. Brasilia. 86 pp.
21. Vainstein, M. H., and Roitman, I. 1985. Cultivation of *Phytomonas* sp. associated with poor development of root system of cassava. J. Protozool. In press.
22. Vermeulen, H. A. 1963. A wilt of *Coffea liberica* in Suriname and its association with a flagellate *Phytomonas leptovassorum* Stahel. J. Protozool. 10:216-222.