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CELL BIOLOGY OF *PHYTOMONAS*, TRYPANOSOMATIDS PARASITES OF PLANTS

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The first decade of this century saw continued interest in diseases of man and animals caused by protozoa belonging to the Trypanosomatidae family. Chagas in 1909 described ***Trypanosoma cruzi***, associating it with a disease subsequently referred to as Chagas' disease. ***T. evansi***, shown to be the agent of surra in horses, was causing trouble in the Mauritius island, where Dr A. Lafont worked as head of the Bacteriology Laboratory (Lafont, 1909).

In 1909, Lafont reported a trypanosomatid in the latex of ***Euphorbia pilulifera***, calling it ***Leptomonas davidi*** in honour of his technician David who first observed the protozoan. These observations were confirmed in the same year by Donovan (1909) who found the same protozoan in ***E. pilulifera*** in India. Donovan proposed the creation of a new genus in the family Trypanosomatidae, naming the protozoan of the latex of ***E. pilulifera, Phytomonas davidi.***

Between 1909 and 1976, there were several reports on the presence of trypanosomatids in the latex of lacticiferous plants including ***Manihot***, a species of economical interest, in the tomato fruits (***Solanum lycopersicum***) (Gibbs, 1957), and in the sieve tubes of plants of economical importance such as ***Coffea liberica*** (coffee) (Stahel, 1931), ***Cocos nucifera*** (coconut) (Parthasarathy et al., 1976), and the oil palm ***Elaeis guineensis*** (Van Slobbe et al., 1978).

The presence of trypanosomatids in plants of economic interest attracted the attention of several research groups. Some of their findings have been reviewed by Dollet (1984), Jankevicius et al. (1988) and Camargo et al. (1990), which emphasized aspects such as life cycle, transmission, and pathogenicity. In this short review we will focus on the cell biology of ***Phytomonas***, discussing aspects related with its cultivation, identification, and morphology.

IDENTIFICATION

All trypanosomatids isolated from plants are considered members of the ***Phytomonas*** genus. Many isolates have been described as new species (***P. davidi, P. leptovasorum, P. tirucalli, P. tortuosa*** and ***P. staheli***). In other cases the trypanosomatids found in plants were described as species of ***Leptomonas*** and ***Herpetomonas*** (Camargo et al., 1990).

The only criterion used to assign these trypanosomatids to ***Phytomonas*** was that they were isolated from plants. Several plants are known to be visited by phytophagous insects which harbour trypanosomatids belonging to different genera. Until we have better criteria for identifying trypanosomatids as belonging to the genus ***Phytomonas*** care should be taken before describing as ***Phytomonas*** any trypanosomatid found in plants. Some authors described as different species, parasites isolated from different plants. Trypanosomatids isolated from one plant species can infect other species. More detailed studies, using morphological, biochemical, and serological studies, should be carried out before establishing new species.

ISOLATION AND CULTIVATION

In the last 30 years our understanding of the biology of trypanosomatids has greatly improved due to the fact that members of the genera ***Trypanosoma, Leishmania, Herpetomonas, Crithidia*** and ***Leptomonas*** could be grown in simple axenic and in chemically defined media. Therefore, getting axenic cultures of ***Phytomonas*** is important in order to improve our knowledge of this genus.

Phytomonads do not grow easilly. McGhee & Postel (1976) isolated a species of ***Phytomonas*** from the latex of ***E. heterophyla***, considering

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it to be *P. davidi*. This isolate has been maintained in axenic media and distributed among several research groups. Until recently it was treated as a true representative of the genus *Phytomonas*. More recently new isolates have been obtained from some Euphorbiaceae (Attias & De Souza, 1986; Dollet et al., 1982; Kastelein & Parsadi, 1988; Vainstein & Roitman, 1987), Apocynaceae (Kastelein & Parsadi, 1988), Arecaceae (Menara et al., 1988), Solanaceae (Jankevicius et al., 1989; Kastelein & Camargo, 1990) and Rutaceae (Conchon et al., 1989). Among these isolates only that from *Manihot esculenta* (Euphorbiaceae) (Aragão, 1927; Vainstein & Roitman, 1987) is pathogenic. An isolate (*P. staheli*) from coconut can be grown using a feeder layer of insect hemocytes and ovocytes (Menara et al., 1988). The growth characteristics of some isolates in various media was determined by da Silva (1990). Members of the genus *Phytomonas* need very rich medium to grow, and they use aerobic metabolism. In addition, this study established for the first time a chemically defined medium in which *P. serpens* (but not the other isolates) can be maintained and showed also that it does not require hemin, a fact observed previously only in those trypanosomatids which harbour an endosymbiont in their cytoplasm (da Silva, 1990).

The obtention of axenic cultures of some phytomonads opened the possibility to carry out some morphological, biochemical and immunological studies providing important informations for a better understanding of the *Phytomonas* genus and by extension of the Trypanosomatidae family.

Da Silva (1990) showed that *P. serpens* requires serine and inositol as essential nutrients and glutamine and glucose as stimulating nutrients. *Phytomonas* does not grow anaerobically and growth is inhibited by KCN.

MORPHOLOGY

Phase contrast microscopy shows that when in the plant most living *Phytomonas* are fusiform organisms twisted two to five times around their longitudinal axis. They measure 10-20 μm in length, have a width of about 1.5 μm. The free (anterior) flagellum measures 10-15 μm. They are often very active. Giemsa-stained preparations show that *Phytomonas* in the laticiferous tubes, in the phloem, and in axenic cultures, are promastigote forms although some paramastigotes can be found. Round amastigotes with or without flagella have been observed in the latex of some plants. Typical opisthomastigotes have been seen in axenic cultures of *P. davidi*. This observation has led some authors to consider this species to be a member of the genus *Herpetomonas* (Camargo et al., 1987, 1990).

Scanning electron microscopy reveals that the surface of *P. davidi* is grooved (Fig. 1). Most of the forms found in the latex of *E. hyssopifolia* are elongated and twisted. Similar observations were made on *P. françai* (Teixeira & Camargo, 1989). Some elongated and shorter parasites from *E. hyssopifolia* were not twisted (Fig. 2).

Many *Phytomonas* with a twisted cell body and a short emerging flagellum were observed in the phloem of the palms *E. guineensis, Roystonea regia* (Fig. 3) and *C. nucifera* (Fig. 4). Most of the protozoa were longitudinally oriented along the main axis of the sieve tube (Fig. 4). Younger sieve tubes are usually more heavily infected than older ones, even when they are contiguous. Quantitative analysis showed that the parasites in the oil palm had a smaller number of twists than those found in coconut. Some untwisted forms were also observed (Figs 3, 4).

Transmission electron microscopy of several *Phytomonas* isolates reveal all structures characteristic of other members of the Trypanosomatidae such as (a) the kinetoplast, (b) subpellicular microtubules, (c) the paraxial rod and (d) glycosomes. There are some differences between various isolates.

Isolates from *E. hyssopifolia* and *E. pinea, P. françai*, and *P. davidi* have a compact kinetoplast DNA network similar to that of most trypanosomatids. The k-DNA network of the isolate from *E. characias* has a loose appearance while the kinetoplast of parasites found in coconut, oil palm, and royal palm show an intermediate appearance (Figs 5 to 8).

The endoplasmic reticulum (ER) has a special organization in some *Phytomonas* isolates. In *P. françai*, the cisternae of the ER form two to four parallel sheets below the pellicular microtubules, longitudinally oriented along the major axis of the cell (Figs 9, 10). Occasionaly the two unit membranes lining the ER cisternae are so close that no intracisternal space is visibe (Fig. 10). In the

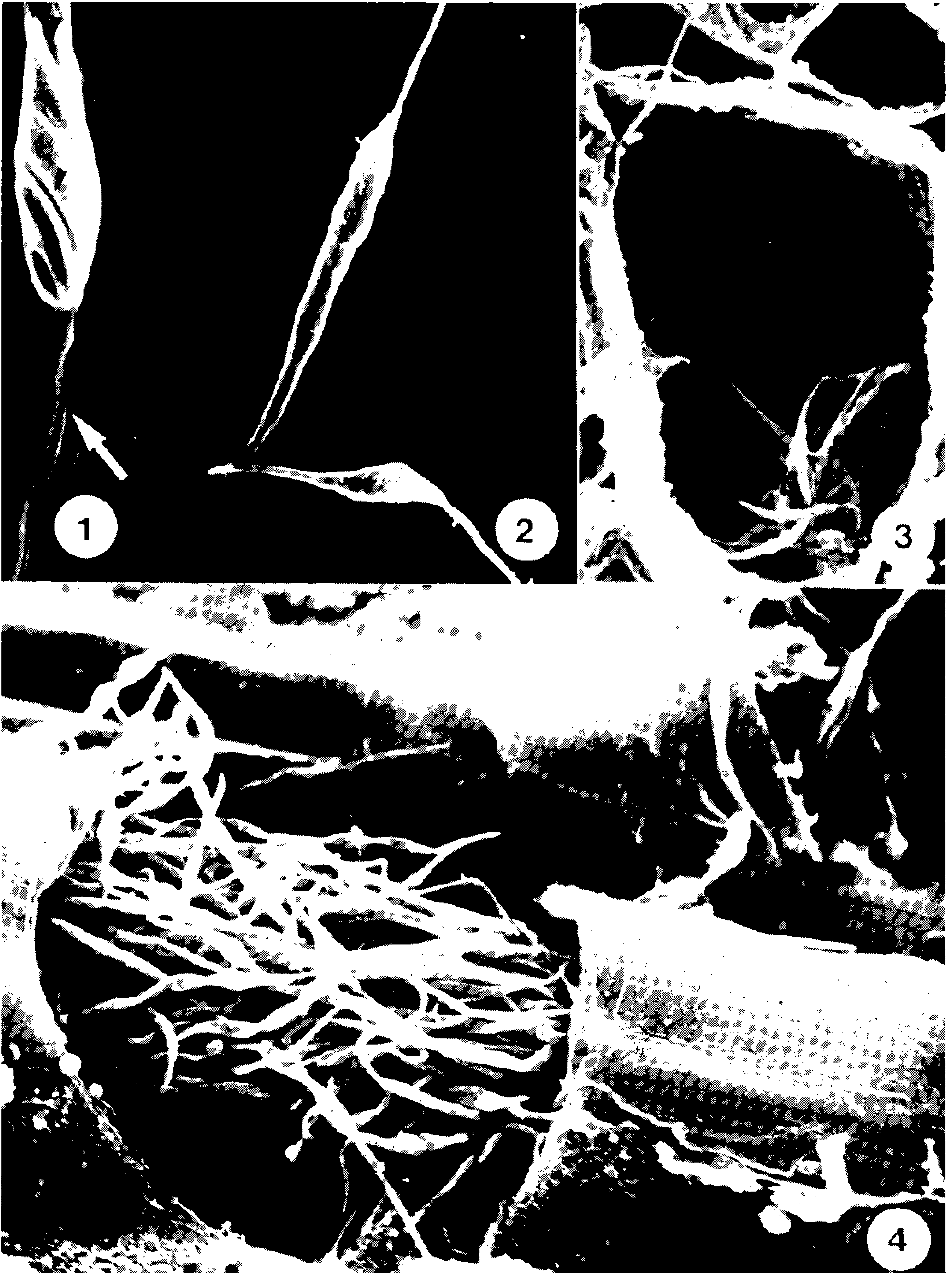


Fig. 1: ***Phytomonas davidi***. Scanning electron micrograph showing a culture form. The cell surface is slightly convoluted forming ridges (arrowheads). The arrow points to the paraxial rod (after Attias & de Souza, 1985). X 8,000. Fig. 2: parasites from the latex from ***Euphorbia hyssopifolia*** infected with ***Phytomonas***. Elongate and short cells lacking surface convolutions are seen (after Attias & de Souza, 1986). X 2,400. Fig. 3: overview of a sieve tube of the stem of ***Roystonea regia*** infected with ***Phytomonas***. Cork screw (arrow) and smooth (asterisk) cells are seen (after Attias et al., 1989). X 1,200. Fig. 4: sieve tube from coconut. Most parasites are longitudinally oriented along the main axis of the sieve tube showing they are twisted and completely obstruct the vessel. X 2,400.

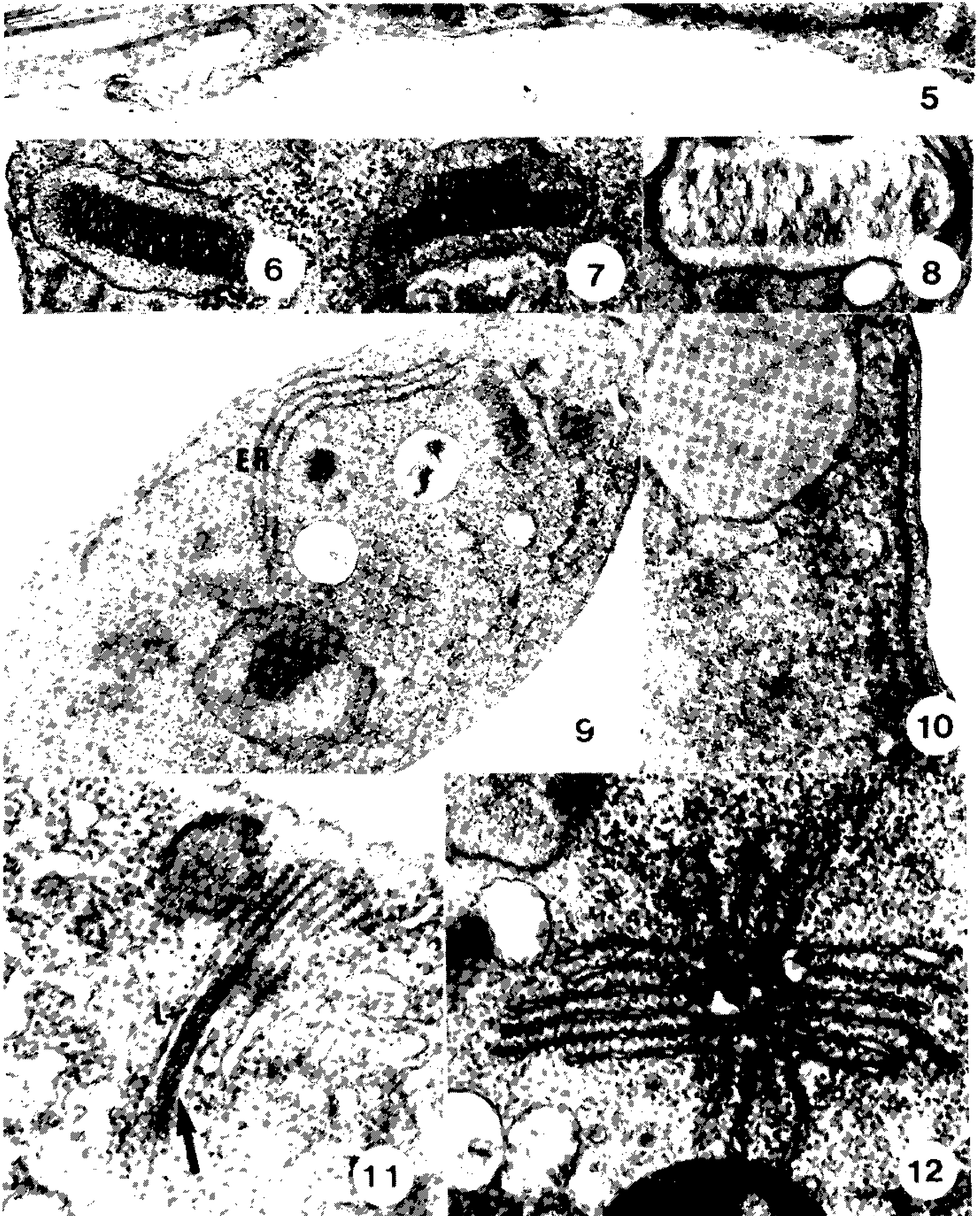


Fig. 5: longitudinal section of the anterior region of ***Phytomonas staheli*** in the phloem of oil palm. The kinetoplast (K) is almost transversally oriented in relation to its usual position in trypanosomatids. The assymmetry of the flagellar pocket is also evident. F. flagellum, N. nucleus, X 22.000 (after Attias et al., 1987). X 17,600. Figs. 6-8: K-DNA pattern in ***Phytomonas*** — Fig. 6: the "compact" pattern, isolate from ***Euphorbia hyssopifolia***. X 32,000. Fig. 7: the double stranded pattern, ***E. hyssopifolia*** isolate. X 32,000. Fig. 8: the loose pattern of the D-DNA network, ***E. characias*** isolate. X 4,000 (after Attias et al., 1988). X 32,000. Figs 9-10: ***Phytomonas françai*** from ***Manihot esculenta***. Fig. 9: shows the kinetoplast (K) and profiles of the endolpasmic reticulum (ER) in parallel rows. X 21,000. Fig. 11: ***Phytomonas*** sp. from ***Euphorbia characias***. There is a paracrystalline array of the ribosomes (arrow) associated with the endoplasmic reticulum. L. lumen of the ER. (after Attias et al., 1988). X 50,000. Fig. 12: ***Phytomonas*** sp. from ***Euphorbia pinea***. The rough endoplasmic reticulum shows a peculiar radiating pattern (asterisk). m. mitochondrion. (after Attias et al., 1988). X 30,000.

Phytomonas sp. from ***E. characias*** and ***E. pinea*** the ribosomes attached to ER have a paracrystalline array (Fig. 11). In many cells of the isolate from ***E. pinea*** various elements of the ER originate from a centripetal focus branching in all directions toward the cell periphery (Fig. 12) where they tend to form layers below the pellicular microtubules.

The glycosome is a specialized type of peroxisome. In the isolate from ***E. hyssopifolia*** the glycosomes occasionally form two rows separated by a bundle of filamentous structures (Fig. 13). The examination of serial sections shows that the glycosome of this isolate is a highly ramified organęlle (Figs 15a, b), as previously observed for example in mouse preputial gland cells (Gorgas, 1984). The peroxisomes of ***E. characias*** may form stacks of flattened disks (Fig. 14). The examination of serial sections revealed that each glycosome in this species was a distinct organelle. The glycosomes occupy about 7.5% of the volume of the isolate from ***E. characias***. This is twice the volume observed for the isolates from ***E. hyssopifolia*** and in ***P. françai***. This suggests that the isolate from ***E. characias*** may be a better model for studies related with carbohydrate metabolism in trypanosomatids.

A contractile vacuole similar to that repported in ***L. collosoma*** (Linder & Staehelin, 1979) is found in the isolates from ***E. hyssopifolia*** and in ***P. davidi***. It is located anteriorly, close to the flagellar pocket. The diameter of the vacuole varies but can reach 2 μm. A spongiome comprised of vesicular and tubular structures with a mean diameter of 50 nm are associated with the large vacuole (Figs 16, 17). This type of spongiome is not usually seen in the other phytomonad isolates nor in several species of ***Herpetomonas, Leishmania*** and ***Trypanosoma*** that we have examined.

CELL SURFACE

The cell surface of trypanosomatids has been subjected to recent review (De Souza, 1989). Lectin-induced agglutination has been used to analyse the sugar residues exposed on the surface of ***Phytomonas***. Lectins such as ***RCA, PNA, WFA*** and ***HPA*** do not agglutinate the isolates form ***E. pinea*** and ***E. characias*** (Petri et al., 1987). Con A, WGA and LPA induce agglutination. Nakamura (1990) reported differences in the pattern of agglutination of several isolates using various lectins.

Several isolates have a net negative surface charge (Esteves et al., 1988; Vommaro et al., 1989) as shown by cell electrophoresis and binding of cationic particles to the cell surface. N-acetyl-neuraminic acid, which in many cells accounts for the negative surface charge, was biochemically detected in ***P. davidi*** (Esteves et al., 1988).

The freeze-fracture technique has been used to analyse the distribution of integral membrane proteins in some ***Phytomonas*** isolates. There are membrane specializations such as the flagellar necklace (Fig. 18) and linear arrays of particles in the membrane lining the flagellar pocket (Fig. 19). The flagellar membrane of the ***Phytomonas*** studied (Fig. 20) have a higher density of membrane particles than that seen in other genera (Attias & De Souza, submitted).

BIOCHEMISTRY

Electrophoretic analysis of isoenzymes show significant differences in band distribution between ***P. françai, P. davidi*** and the isolates from ***E. hyssopifolia, E. pinea*** and ***E. characias*** for glucose-phosphate isomerase (GPI), malate dehydrogenase (ME), arpartate aminotransferase (ASAT), alanine aminotransferase (ALAT), phosphoglucomutase and glucose-6-phosphate dehydrogenase (G6PD). The isolates from ***E. pinea*** and ***E. characias*** have the same isoenzyme pattern for ALAT, ASAT and G6PD. For all enzymes tested, marked differences have been observed between the isolates from ***E. hyssopifolia*** and ***P. davidi*** (Vainstein et al., 1987). Therefore, isoenzyme profiling clearly distinguishes the various isolates examined up to now and confirms their distinctiveness.

The ornithine-arginine metabolic pathway in trypanosomatids has been extensively investigated by Camargo et al. (1987), providing informations which, besides their biochemical significance, help in the identification of trypanosomatid genera. None of the isolates of ***Phytomonas*** examined possesses arginase. This also applies to trypanosomatids of the genus ***Herpetomonas***, and permits their distinction from the genera ***Leishmania, Leptomonas*** and ***Crithidia***.

The kinetoplast DNA network of ***Phytomonas*** sp. from ***E. pinea*** represents more than 30% of the total cell DNA, the highest proportion of k-DNA found in any trypanosomatid. It is

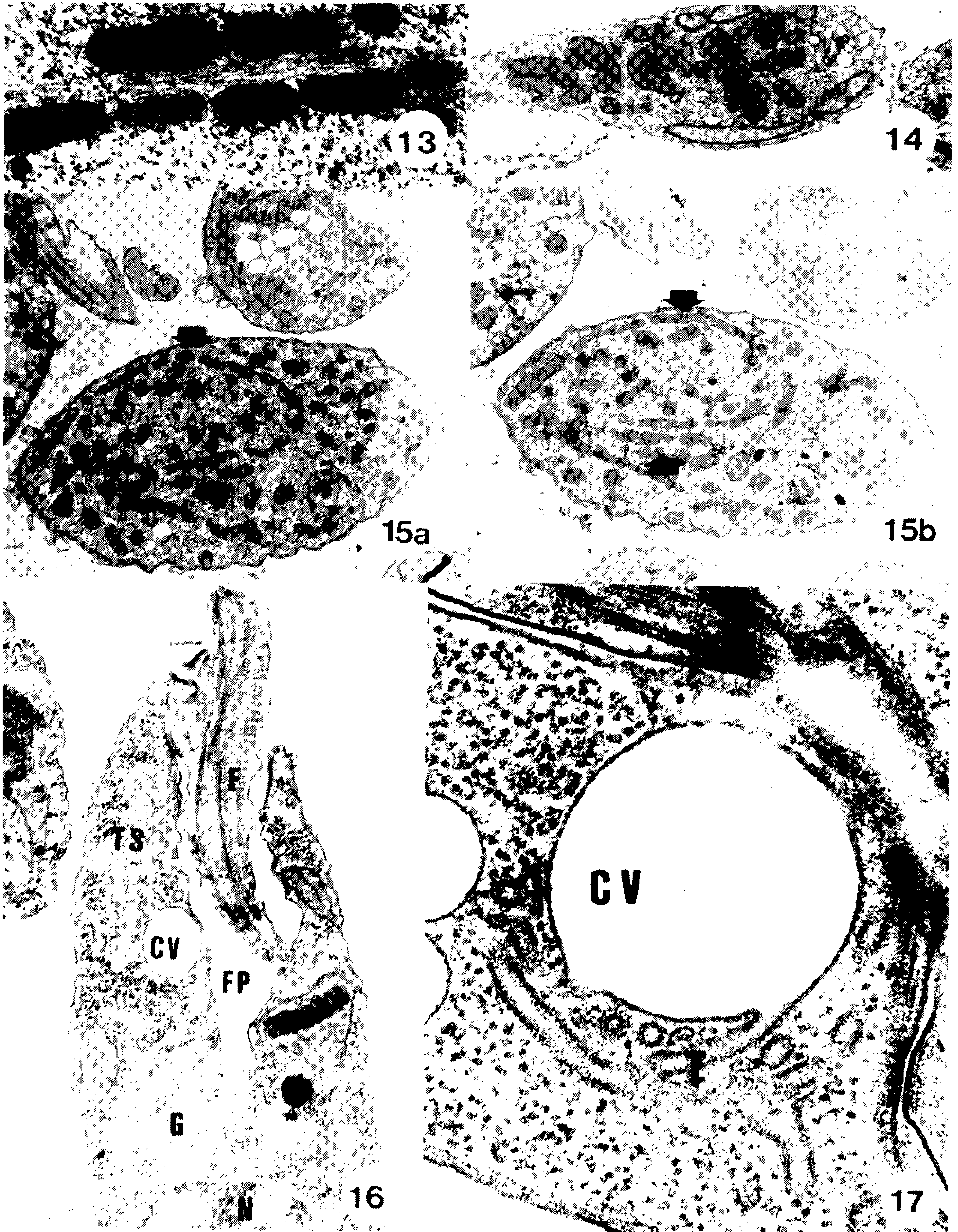
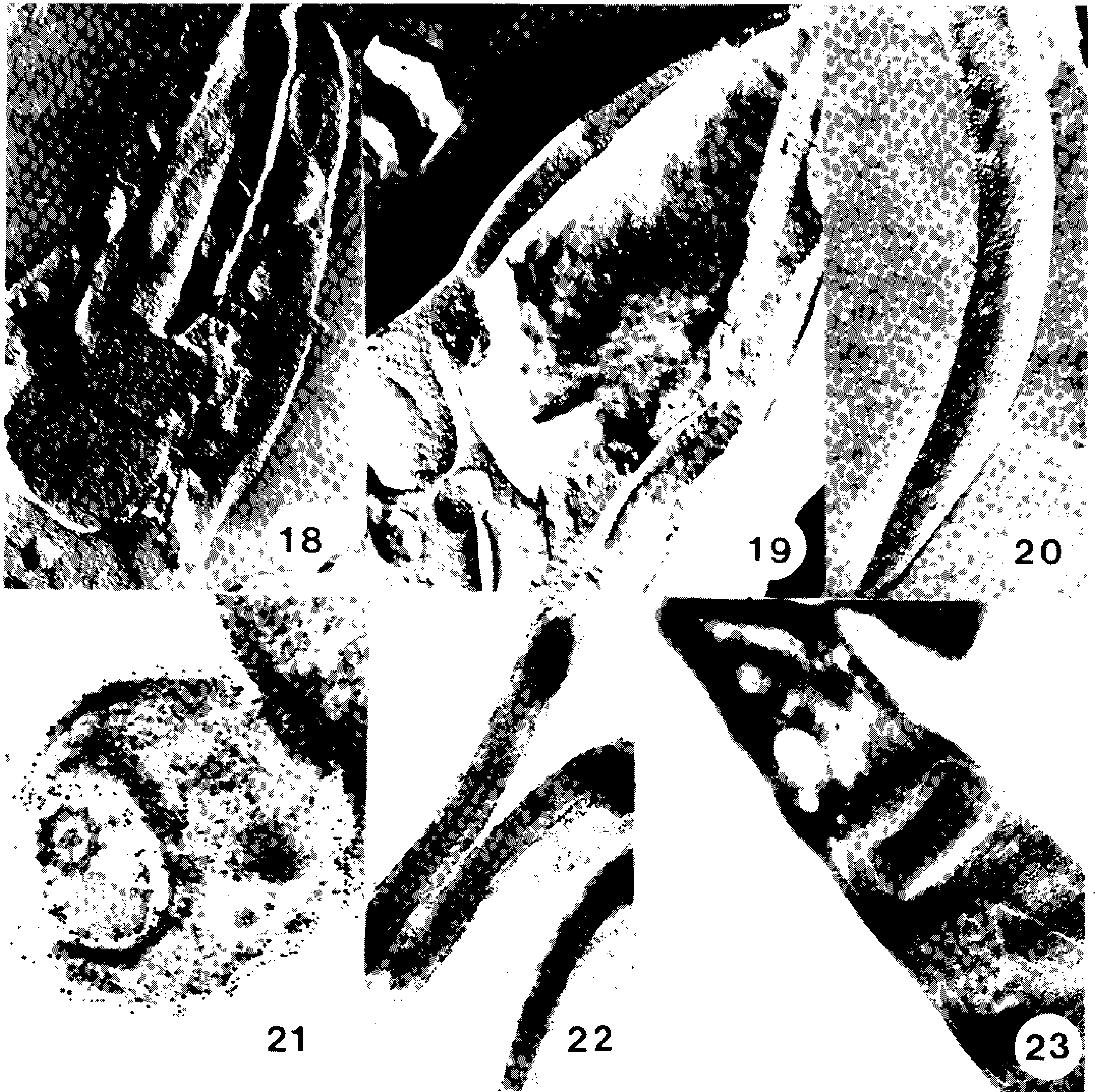


Fig. 13: ***Phytomonas*** sp. from ***Euphorbia hyssopifolia*** Peroxisomes (P) are arranged in two rows separated by a bundle of filamentous structures (arrows). (after Attias et al., 1988). X 28,000. Fig. 14: ***Phytomonas*** sp. isolated from ***Euphorbia pinea*** disposed in stacks of flattened organelles. (after Attias et al., 1988). X 12,800. Figs 15a, b: serial sections of the ***Euphorbia hyssopifolia*** isolate. Arrows point to fusion of apparently independent peroxisomes. The asterisk shows the germinative zone of the multibranched organelle. (after Attias & de Souza, submitted). X 10,000. Fig. 16: longitudinal section through the anterior region of ***Phytomonas davidi***. FP. flagellar pocket, F. flagellum, CV. contractile vacuole, TS. contractile vacuole tubular system; G: Golgi complex; N: nucleus; K: kinetoplast (after & De Souza, 1985). X 18,400. Fig. 17: contractile vacuole from ***Phytomonas davidi*** showing fusion between the collecting tubules (T) and the central vacuole (CV) (after Attias & de Souza, 1985). X 48,000.



Figs 18-20: freeze-fracture replicas (from Attias & de Souza, submitted). Fig. 18. ***Phytomonas françai*** general view showing the ciliary necklace (arrowhead), kinetoplast (K) localization is also apparent. X 24,000. Fig. 19: intramembrane particles form regular arrays (arrow) on the flagellar pocket membrane of a cell from the ***Euphorbia pinea*** isolate. X 19,200. Fig. 20: flagellar membrane of the ***E. pinea*** isolate. Particles are randomly distributed on the E face. X 22,500. Figs 21-23: immunolabelling of thin sections of ***Phytomonas*** embedded in Lowicryl and incubated in the presence of antibodies raised against ***Phytomonas*** sp. from ***Euphorbia hyssopifolia***. Gold particles are seen along plasma and flagellar membrane. Fig. 21: ***P. davidi***. X 52,000. Fig. 22: labelling of flagellar membrane in the ***E. hyssopifolia*** isolate. X 24,000. Fig. 23: no labelling is observed in ***P. françai*** after incubation with the anti ***E. hyssopifolia*** isolate serum. X 17,600.

composed of catenated large minicircles (about 7000 minicircles of 2880 ± 30 base pairs each). These are not cleaved by some restriction enzymes usually employed such as EcoRI, Hae III, Bam HI and Hind III. They are cleaved by Hpa II. The minicircles have little sequence homology with those of other trypanosomatids. Total k-DNA from *P. serpens* isolated from tomatoes and from the insects *Phthias picta* and *Nezara viridula* cross hybridized among the various strains but linearized minicircles of 1.4 kilobases hybridize only with the homologous strain. From 20 different restriction enzymes tested, only five were able to digest the k-DNA of *P. serpens* confirming its relative stability. More recently from as study of eleven isolates of *Phytomonas* from phloem and latex,

Riou and co-workers concluded that they may be grouped in three categories according to minicircle size, restriction endonuclease cleavage and sequence homology (Riou et al., 1987).

SEROLOGY

Some attempts have been made to identify antigens in some isolates using monclonal and polyclonal antibodies in agglutination, immunoprecipitation, immunofluorescence, and immunocytochemistry. It is possible to distinguish isolates according to their geographical origin and to separate *Phytomonas* isolates from the south of France from other lower trypanosomatids (Petry et al., 1989). Monoclonal antibodies raised against *P. français* or *P. serpens* labelled *P. serpens* and isolates from *E. pinea, Jathropa macrantha* (Euphorbiaceae) and *Allamanda cathartica* (Apocynaceae) (Teixeira & Camargo, 1989). Some antibodies recognize only *P. français*; only one recognized the isolate from *E. hyssopifolia* and *E. characias*. None of the antibodies recognized isolates of *Leptomonas, Crithidia, Trypanosoma* and *Leishmania*, nor *P. davidi*. Attias and De Souza (submitted) analyzed five *Phytomonas* isolates. It was possible to separate them into two groups using the following techniques) (a) SDS-PAGE analysis; isolates from *E. hyssopifolia* and *P. picta* and *P. davidi* have the same protein profile which is distinct from that of the isolates from *E. characias, E. pinea* and *P. français*; (b) immunoblotting analysis of two polyclonal antibodies against the isolate from *E. hyssopifolia* or *P. français* produced two labeling patterns with the antigens located in the cell membrane and in a less extent, in the cytoplasm (Figs 21-23).

LIFE CYCLE OF *PHYTOMONAS*

We do not have a clear idea about the complete life cycle of *Phytomonas*. In the plants, the promastigote form predominates, despite the presence of some rounded forms with a short flagellum. Another part of the cycle takes place in the insect (Dollet, 1984). In *Oncopeltus* spp. infected with *P. elmassiani* a giant form is found in the pylorus 8 days after infection. Later, small forms are observed. The parasites reach the hemolymph, where giant forms are seen, and subsequently the salivary glands, where giant and small forms coexist (McGhee & Hanson, 1969; Vickerman, 1962). Similar observations have been made in other insects (Jankevicius et al., 1989). In *Phthias picta* small rounded forms of *P. serpens* are found mainly in the urine and long typical promastigotes in the digestive tract. No giant forms were described (Jankevicius et al., 1989). Further studies using electron microscopic techniques are necessary for a better understanding of the *Phytomonas* — insect relationship.

CONCLUSION

Some members of the *Phytomonas* genus are pathogenic, affecting plants of great economic interest such as coffee, coconut, oil palm, cassava, tomato, soy-bean, and others. Before we can make progress in this area of phytopathology we need (a) to develop more adequate media to culture these protozoa, (b) to analyze the effects of the presence of the parasite on the plant's metabolism, (c) to understand the life cycle of the parasite especially in the invertebrate host, and (d) determining the mechanisms by which the parasites exert their pathogenic effect on the plants.

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