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Trypanosomatid protozoa in plants of southeastern Spain: characterization by analysis of isoenzymes, kinetoplast DNA, and metabolic behavior

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Abstract Three flagellates of the family Trypanosomatidae were isolated from mango fruits (*Mangifera indica*) and from the stems of clover (*Trifolium glomeratum*) and Amaranth (*Amaranthus retroflexus*) in southeastern Spain and were adapted to in vitro culture in monophase media. The parasites showed an ultrastructural pattern similar to that of other species of the genus *Phytomonas*. Mango and clover isolates differed from amaranth isolates in ultrastructural terms. The isolates were characterized by isoenzymatic analysis and by kDNA analysis using five different restriction endonucleases. With eight of the nine enzymatic systems, mango and clover isolates were distinguished from those of amaranth. Nevertheless, with the enzymes malate dehydrogenase and superoxide dismutase, flagellates isolated from clover were differentiated from those isolated from mango. Electrophoretic and restriction-endonuclease analysis of kDNA minicircles showed similar restriction cleavage patterns for the isolates from mango and clover, whereas the patterns of the amaranth isolates differed. The results of the present study confirm that the strains isolated from mango and clover constitute a phylogenetically closely related group of plant trypanosomatids, which is more distantly related to the strain isolated from amaranth. The similarities in the results obtained for isolates from mango and clover foliage, on the one hand, and those obtained from tomato and cherimoya fruits (studied previously), on the other, as well as the geographic proximity of the different plants support the contention that only one strain is involved, albeit one strain that can parasitize different plants. Furthermore, some of the plants appear to act as reservoirs for the parasites. On the other hand, the metabolism studies using [^{1}H]-nuclear magnetic resonance spectroscopy did not reveal that the catabolism of *Phytomonas* in general follows a pattern common to all the species or isolates. *Phytomonas* are incapable of completely degrading glucose, excreting a large part of their carbon skeleton into the medium as fermentative metabolites (acetate, ethanol, glycine, glycerol, and succinate).

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Introduction

It has been known since 1909 that plants may be parasitized by members of the family Trypanosomatidae, particularly by species of *Phytomonas*, a genus arbitrarily created by Donovan (1909) to differentiate plant trypanosomatids from those that parasitize animals. This kind of trypanosomatid is found over a wide range of geographical areas, including northern and central Africa and, in the Western hemisphere, in several European countries such as France, the Czech Republic, and the Russian Federation (Dollet 1984; Camargo et al. 1990).

Recently our research team achieved the isolation and in vitro culture of flagellates from tomato and cherimoya fruits grown in southeastern Spain (Sánchez-Moreno et al. 1995a). In that and other studies (Muller et al. 1994; Fernández-Becerra et al. 1996) we found that *Phytomonas* isolated from different fruits have a common origin and that the same flagellate can parasitize various plants.To test this hypothesis we sampled other crops of economic importance in southeastern Spain and also sampled wild plants near the crops to determine whether these could serve as reservoirs for the parasites.

In the present paper we describe flagellate isolates from the fruit of *Mangifera indica* and from the stems of *Trifolium glomeratum* and *Amaranthus retroflexus* in

southeastern Spain. One major interest of this study was the considerable decline in fruit and vegetable production in our region in recent years, representing an important economic issue. After isolation and in vitro culture of these flagellates we conducted ultrastructure studies, and three isolates were characterized by isoenzyme studies, by the analysis of kDNA restriction-fragment-length polymorphism using different restriction endonucleases, and by their metabolic behavior using proton nuclear magnetic resonance.

Materials and methods

Plant survey

Vegetative and fruit samples from trees of *Mangifera indica* as well as wild plants of *Trifolium glomeratum* and *Amaranthus retroflexus* were collected along the southern coast of Spain. Small sections of the fruits, roots, stems, and leaves were homogenized in phosphate-buffered saline (PBS) and examined by phase-contrast microscopy. Sample smears were fixed in methanol and stained with buffered Giemsa (pH 7.0) for light microscopy.

In vitro culture

The samples found to be infected with *Phytomonas* were inoculated in 3 ml of Grace's (Gibco) medium supplemented with 10% heat-inactivated fetal calf serum in 25-ml Falcon flasks. Cultures cleaned of contaminants were kept at 28 °C in an antibiotic-free medium. Aliquots of medium and cells were collected for growth measurements. Growth was assessed by cell counting in a Neubauer hemocytometer and by light microscope studies of cells fixed in methanol and stained with buffered Giemsa (pH 7.0).

Electron microscope observation

Parasites cultured in Grace's medium as previously described were collected during the exponential growth phase by centrifugation at 1500 *g* for 10 min and were fixed for 2 h in a 2.5% (v/v) solution of cacodylate-buffered 0.1 *M* glutaraldehyde containing 0.1 *M* $CaCl_2$ and 0.9% NaCl (Sánchez-Moreno et al. 1995a). Fixed cells were washed in the same buffer and postfixed in isotonic saline solution containing 1% (v/v) osmium tetroxide and 0.08% potassium fluorocyanide. The cells were then washed in buffer, dehydrated in acetone, and washed and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and then examined with an EMCIO Zeiss transmission electron microscope.

Isoenzyme analysis

For the preparation of homogenates, 25 ml of culture medium with a cell density of 2×10^7 cells ml^{-1} were centrifuged at 1500 *g* for 10 min; washed twice in PBS (pH 7.0); resuspended in a hypotonic enzyme stabilizer containing 2 m*M* dithiothreitol, 2 m*M* *E*-aminocaproic acid, and 2 m*M* ethylenediaminetetraacetic acid (EDTA; Fernández-Becerra et al. 1996); and, finally, frozen at −80 °C for 15 min and then thawed at 25 °C, this process being repeated several times. Next, cell lysates were centrifuged at 8000 *g* for 20 min at 4 °C, and the supernatants were stored in liquid nitrogen until used. The protein concentration was determined by the Bio-Rad method (Bradford 1976) and the samples were stored at a final concentration of 1 mg (ml of protein)$^{-1}$.

The enzymes were separated by isoelectric focusing in a PhastSystem apparatus (Pharmacia-LKB) using Phas-gel IEF 3-9 (Pharmacia-LKB; Fernández-Becerra et al. 1996). The following enzymes were tested: malic enzyme (ME; E.C.1.1.1.40.), malate dehydrogenase (MDH; E.C.1.1.1.37.), glutamate dehydrogenase (GDH; E.C.1.4.1.3.), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; E.C.1.2.1.12.), isocitrate dehydrogenase (IDH; E.C.1.1.1.42.), glucose phosphate isomerase (GPI; E.C.5.3.1.9.), phosphoglucomutase (PGM; E.C.2.7.5.1.), and adenylate kinase (AK; E.C.2.7.4.3.). The staining procedures have been described by Ben Abderrazak et al. (1993). Superoxide dismutase (SOD; E.C.1.15.1.1.) was assayed as described by Beyer and Fridovich (1987).

►

Fig. 1a–f Sections of flagellates isolated from plants maintained in axenic cultures. Isolates from *Trifolium glomeratum* (**a** ×32,000; **b** ×63,000), from *Mangifera indica* (**c** ×14,000; **d** ×20,000) and from *Amaranthus retroflexus* (**e** ×20,000; **f** ×63,000). (*MT* Pellicular microtubules, *M* mitochondion, *K* kinetoplast, *N* nucleus, *NU* nucleolus, *FP* flagellar pocket, *FA* flagellar axoneme, *R* ribosome, *ER* endoplasmic reticulum, *G* glycosomes, *LG* lipid globules, *FS* flagellar section, *PR* paraxial rod)

Kinetoplast DNA isolation, restriction enzyme digestion, and electrophoretic analysis

Cells were collected by centrifugation of 300 ml of culture medium after about 5 days, when their concentration had reached about 2×10^7 cells ml^{-1}, and were washed twice in 50 ml of 0.15 *M* NaCl, 0.015 *M* Na citrate and once with SE buffer (0.15 *M* NaCl, 0.1 *M* EDTA, pH 8.0). Kinetoplast DNA (kDNA) was obtained according to the procedure described by Goncalves et al. (1984).

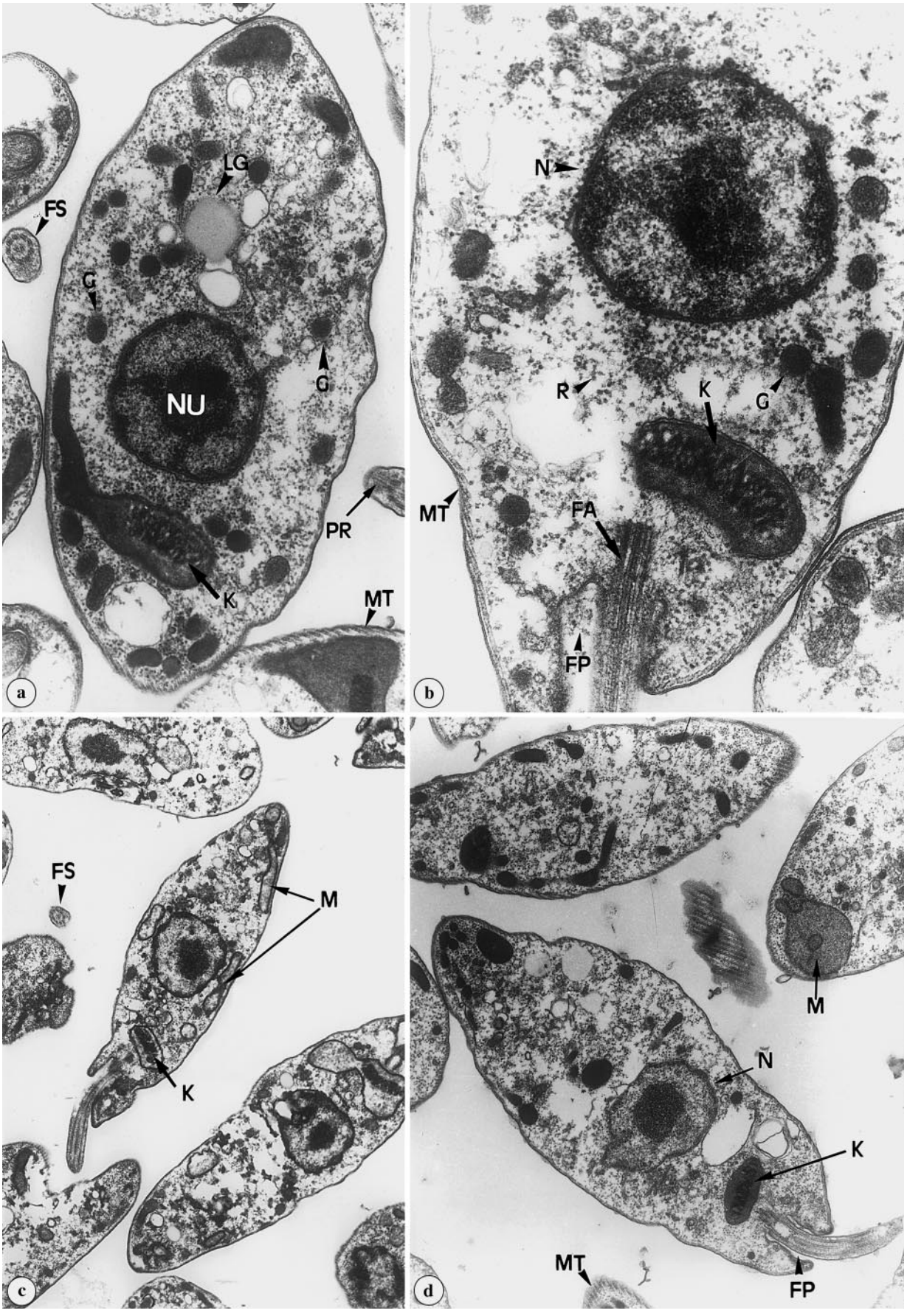
The kDNA extracts (1 mg ml^{-1}) were completely digested with restriction endonucleases (MspI/HpaII, AluI, BmyI, HaeIII, and HinfI) according to the manufacturer's prescribed buffer conditions (Boehringer). The digestion products were electrophoresed in 1.5% agarose slab gels as described by Riou and Yot (1977), and the fragment sizes were estimated by comparison of their mobilities with those of a 1-kb DNA ladder (Gibco BRL). The gels were stained with ethidium bromide (10 mg ml^{-1} for 10 min) and photographed under UV light with a Polaroid camera (665 film).

Metabolism study by [^{1}H]-nuclear magnetic resonance

Cell cultures were started by inoculation of 1 ml (approximately 1×10^6 cells/ml) of the three new isolates of *Phytomonas* culture in the logarithmic growth phase in 70 ml of Grace's medium in 250-ml Falcon flasks. On each of the following 5 days a 10-ml aliquot was taken and the amount of parasites was determined in a Neubauer hemocytometer chamber, after which the cells were removed by centrifugation at 600 *g* for 10 min. The pH of the parasite-free supernatants was measured before they were frozen at −80 °C for metabolite determination by [^{1}H]-nuclear magnetic resonance ([^{1}H]-NMR) spectroscopy. Grace's medium that had not been inoculated was used as a control.

The [^{1}H]-NMR spectra were obtained with a Bruker AM-300 spectrometer operating at 300.13 MHz (Gilroy et al. 1988). The temperature of the probe was maintained at 27 °C. The pulse technique and Fourier transformation were used with 90 °C pulses and a sweep width of 3287.5 Hz. The water signal was eliminated by presaturation, irradiating at water frequency for 2.5 s selectively with intervals between pulses of 8 recycle times and 160 accumulations. Chemical shifts are expressed as parts per million relative to tetramethyl xylane.

The chemical shifts used to identify the respective metabolites were in agreement with those reported in the literature (Sánchez-Moreno et al. 1995b). The resonances of a given number of metabolites were assigned by the addition of pure components to the fresh medium, and the changes in respective peak intensity were then measured.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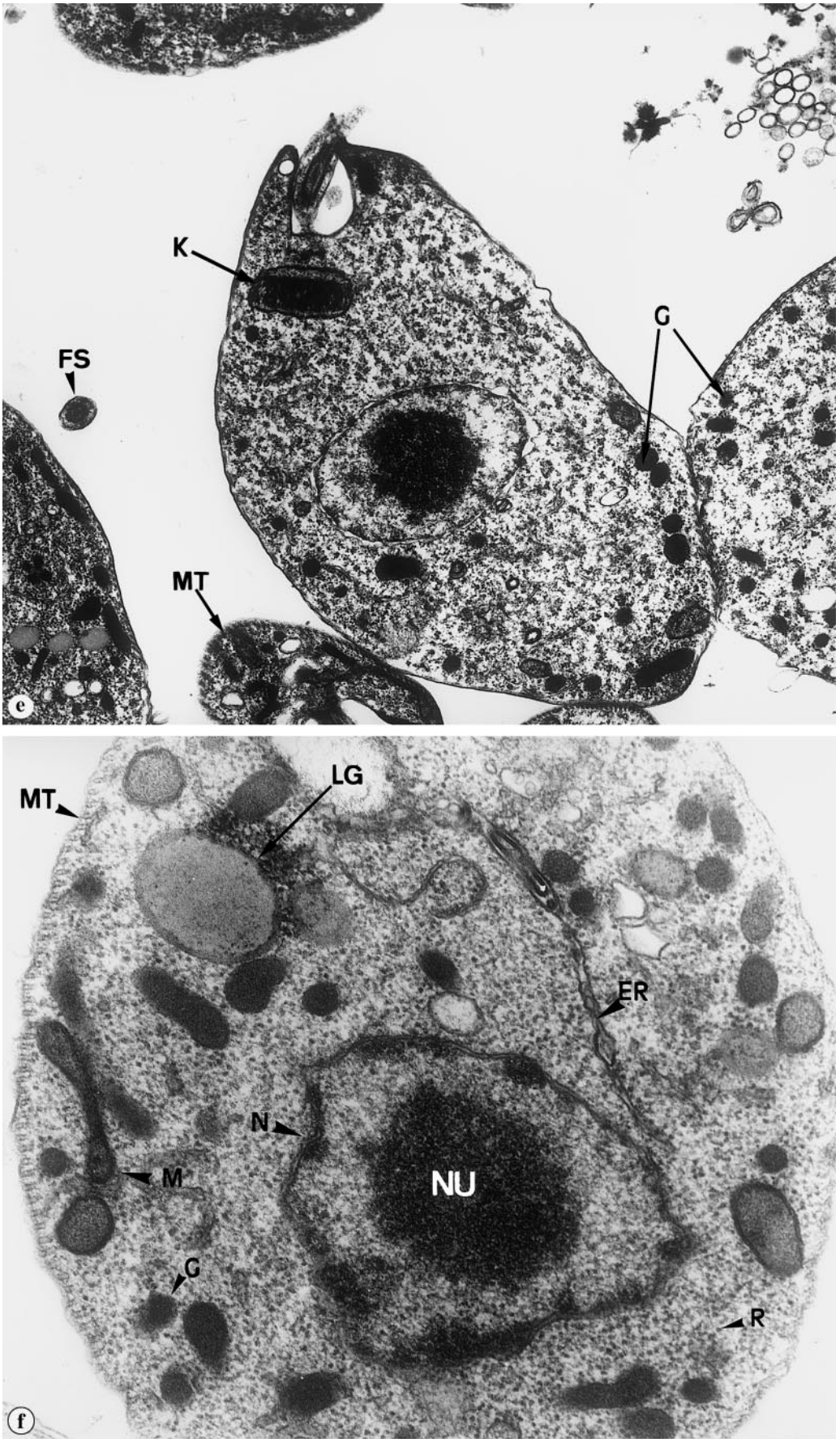

Fig. 1a–f

Results

Sampling several provinces in southeastern Spain, we found trypanosomatid flagellates in the fruit of *Mangifera indica* trees and in the stems of wild *Trifolium glomeratum* and *Amaranthus retroflexus*. The latter two plants grow close to cherimoya trees, where we had previously detected flagellates (Sánchez-Moreno et al. 1995a). In the clover and amaranth plants the flagellates were invariably found in the stems.

The mango trees showed signs of an unidentified sickness, an overall pseudochlorosis, leaf loss, and reduction in the number of fruit, which were deformed and tended to fall before they were fully ripe; in less than 3 years the trees died. The clover and amaranth plants containing flagellates were found growing much like weeds very near to diseased cherimoya trees.

The mango tree samples, examined under a light microscope, revealed no trypanosome in phloem or other tissues in the leaves, petioles of leaves, or stems. Instead the flagellates were situated immediately under the pericarp of the fruit.

The flagellates, stained with Giemsa and observed under a light microscope, appeared in the promastigote form. In all three cases the flagellates were highly polymorphic, with large and smaller forms having a flagellum, resembling in both size and shape the pathogens detected in other fruits (Attias et al. 1988; Jankevicius et al. 1989; Sánchez-Moreno et al. 1995a).

The three new isolates were adapted to in vitro culture using Grace's medium. In the first cultures the mobility of the parasites was perceptible at a few hours after seeding. In successive subcultures the number of parasites per volume unit increased, as did mobility, reaching growth densities of 10^7 cells/ml in the stationary phase after several subcultures. Growth was exponential during the first few days of culture, becoming stationary on day 6 (data not shown).

The flagellates isolated from amaranth plants had a more globular and polymorphic form than did the isolates from mango and clover, the body size of all these isolates ranging from 6 to 14 mm, with a flagellum of 7–10 mm.

The electron microscope studies revealed certain ultrastructural differences between the flagellates isolated from clover (Fig. 1a, b) and mango (Fig. 1c, d) with respect to the amaranth isolates (Fig. 1e, f). The latter had fewer lipid vacuoles than the mango and clover isolates, which were similar in this respect. The mitochondrial profile of the mango and clover flagellates was oval and adapted to the size of the kinetoplast, showing a highly dense, elongated mitochondrial matrix that was boomerang-shaped in cross section. In mango isolates the peripheral sections of the mitochondria were more sparse, whereas amaranth flagellates showed a mitochondrial cross section that was not adapted to the size of the DNA, was not dense, and had elongated peripheral sections.

In the mango and clover isolates the endoplasmic reticulum was practically imperceptible, although in the case of the clover isolates the smooth endoplasmic reticulum contained scattered lacunae, which were much more abundant in amaranth. In the mango, clover, and amaranth flagellates the glycosomes were rounded or oval, highly electron-dense, and dispersed in the former two plant isolates, whereas they were much more abundant and were located along the periphery in the amaranth flagellates.

Using nine enzymatic systems, we could differentiate between the three isolates of this study. As shown in Fig. 2, the isoenzyme profiles for each sample were given both by the number of bands and by their mobility on being separated electrophoretically according to their isoelectric point. With eight of the nine enzymatic systems tested (GAPDH, GDH, IDH, MDH, ME, GPI, PGM, and SOD) we differentiated the isolates of mango (lane 1) and clover (lane 2) from those of amaranth

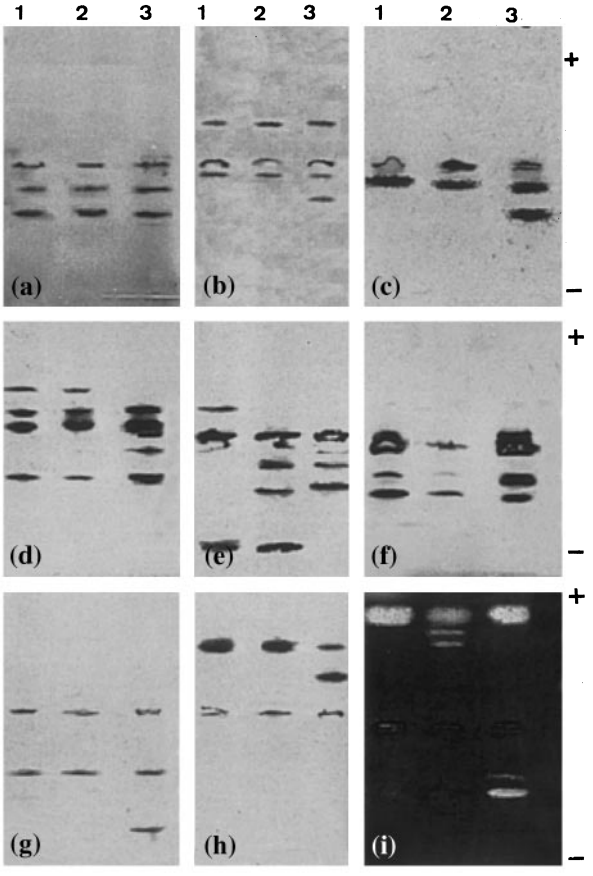

Fig. 2a–i Isoenzyme profiles of different *Phytomonas* stocks. Organisms: *M. indica* (*lane 1*), *T. glomeratum* (*lane 2*), and *A. retroflexus* (*lane 3*). Enzymes: **a** adenylate kinase (*AK*), **b** glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), **c** glutamate dehydrogenase (*GDH*), **d** isocitrate dehydrogenase (*IDH*), **e** malate dehydrogenase (*MDH*), **f** malic enzyme (*ME*), **g** glucose phosphate isomerase (*GPI*), **h** phosphoglucomutase (*PGM*), and **i** superoxide dismutase (*SOD*)

plants (lane 3). Nevertheless, only with the enzymes MDH and SOD could we differentiate the clover from the mango foliar flagellates. The electrophoretic profile presented by the mango isolates for the enzyme MDH was composed of three bands, whereas that of the clover isolates had four bands (Fig. 2e). For the enzyme SOD the clover isolates presented a highly intense band with a pI of 8.5, as did the other two isolates, and a series of less intense bands near a pI of 8 that were not detected in the electrophoretic profile of the other isolates (Fig. 2i).

The other technique used for characterization was kDNA analysis after digestion with restriction enzymes. For this purpose, we purified the kDNA of the three isolates and treated it with five restriction enzymes (Bmy I, Hae III, HinfI, AluI, and MspI/HpaII). Electrophoresis performed in agarose gels revealed that the undigested kDNA could not penetrate the gel (Fig. 3, lane 17). Not all the enzymes used were capable of digesting the kDNA of the isolates; that is, BmyI could not digest the kDNA of any of the three isolates (Fig. 3, lanes 2–4) and HaeIII was incapable of cutting the kDNA belonging to the amaranth isolates, the genetic material remaining in the upper part of the gel in both cases (Fig. 3, lane 7). In all other cases, digestion was complete, providing numerous kDNA fragments.

The three isolates were considerably heterogeneous with respect to the minicircles, although this diversity was more accentuated in the strains isolated from mango (Fig. 3, lanes 2,5,8,11, and 14) and clover (Fig. 3, lanes 3,6,9,12, and 15) than in those isolated from amaranth (Fig. 3,4,7,10,13, and 16). The restriction patterns also revealed the presence of faint bands, which corresponded to molecules exceeding 5 kb in molecular weight (Fig. 3, lanes 5 and 6).

To identify the principal metabolites excreted by the flagellates cultured for 5 days on Grace's medium, we analyzed the medium by [^{1}H]-NMR spectroscopy. An example of the [^{1}H]-NMR spectra obtained with Grace's medium is shown in Fig. 4. Figure 4a illustrates the spectrum obtained with fresh (uninoculated) medium, and Fig. 4b depicts the spectrum yielded by cell-free medium at 5 days after inoculation with the flagellate isolates. Additional peaks, corresponding to the major metabolites produced and excreted during growth, were distinguished in a comparison with fresh medium. Acetate (1.90 ppm), ethanol (1.20 ppm), glycine (3.63 ppm), glycerol (3.98 ppm), and succinate (2.40 ppm) were the metabolites excreted by the *Phytomonas*. On comparing the spectra obtained from the different isolates, we found differences in the height of the peaks. Data for the clover and amaranth isolates are not shown because their spectrum was very similar to that of the mango isolates.

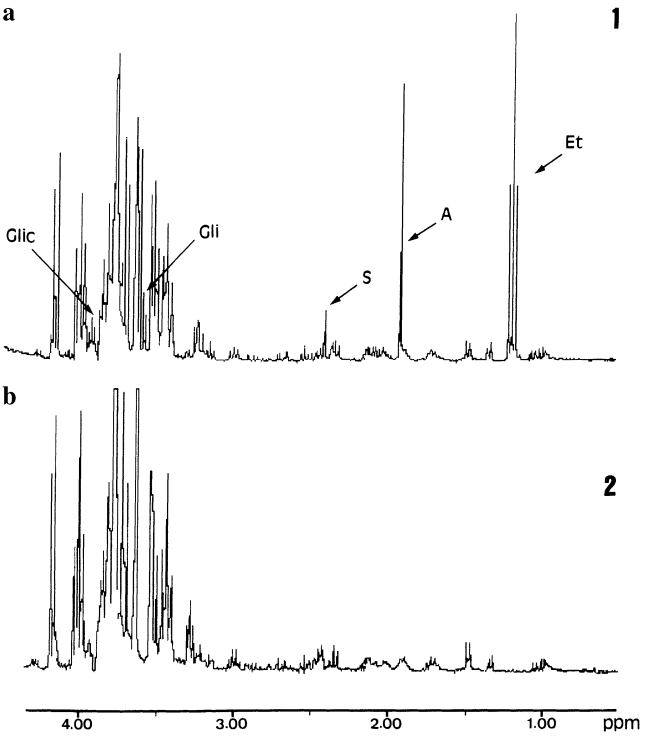

Fig. 4a, b NMR spectra of flagellates isolated from *Mango* sp. medium, showing metabolite production and utilization during normal growth in Grace's medium. **a** Fresh medium. **b** After 5 days of culture. Metabolites excreted and chemical shifts were: *A* acetate (1.90 ppm), *Et* ethanol (1.20 ppm), *Glic* glycerol (3.98 ppm), *Gli* glycine (3.63 ppm), and *S* succinate (2.40 ppm)

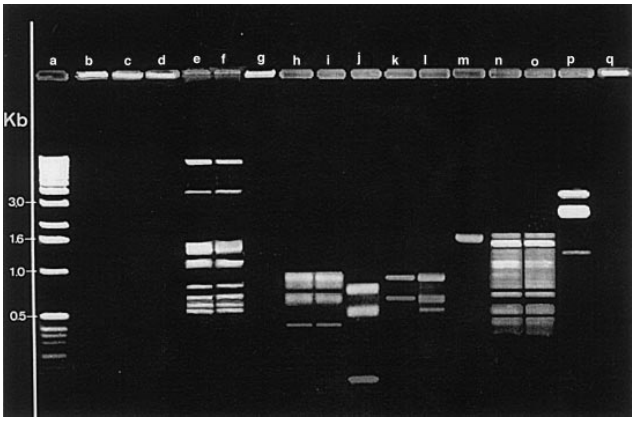

Fig. 3 Restriction endonuclease analysis of kDNA from *Phytomonas*: *M. indica* (*lanes 2,5,8,11,14*), *T. glomeratum* (*lanes 3,6,9,12,15*) and *A. retroflexus* (*lanes 4,7,10,13,16*). Lanes: *2–4* kDNAs + BmyI, *5–7* kDNAs + HaeIII, *8–10* kDNAs + HinfI, *11–13* kDNAs + AluI, *14–16* kDNAs + HpaII. Size markers are 1-kb DNA-ladder fragments (*lane 1*). Intact kDNA is depicted in *lane 17*

Discussion

We are currently proceeding with epidemiology studies based on other cultures and also sampling wild plants near the crops to ascertain whether these could serve as reservoirs for the flagellates. Culturing isolates in vitro, we found flagellates in mango fruits and in the stems of clover and amaranth plants.

Under a transmission electron microscope these forms revealed an ultrastructural pattern typical of trypanosomatids. All basically presented a pellicular membrane reinforced by evenly disposed subpellicular microtubules, a mitochondrion, a kinetoplast, a double nuclear membrane with nuclear pores, a nucleolus, a basal body, a flagellar pocket, a flagellar sheath, an axoneme, and a paraxial rod.

The ultrastructure studies enabled us to discern certain differences in the flagellates isolated from amaranth plants with respect to the flagellates isolated from mango and clover, suggesting that these represented two different strains. The ultrastructure of the latter two types closely resembles that of isolates from tomato and cherimoya fruits (Sánchez-Moreno et al. 1995a).

As these ultrastructural differences could be due to the different physiological stages of the organisms, morphological criteria are inadequate to distinguish strains, nor does the parasitism of different species of host plants signify different parasite strains (Conchon et al. 1989). As a result, it is necessary that thorough biochemistry studies be carried out to establish reliable taxonomic criteria.

To characterize the plant trypanosomatids in the present study we electrophoretically analyzed the isoenzyme by isoelectric focusing (Salas et al. 1995). This method enabled us to distinguish flagellates that had been isolated from different hosts, although the differences in the isoenzymatic profiles of the mango and clover flagellates were less pronounced with respect to each other than with respect to the amaranth isolates. Comparing the isoenzymatic profiles obtained for mango and clover with those obtained for tomato and cherimoya fruits isolated previously (Fernández-Becerra et al. 1996), we found a strong similarity, which leads us to conclude that the isolates displaying these profiles have a common identity.

To test this conclusion we compared the different kDNA fragments obtained with different endonucleases. The kDNA of the three isolates used was purified and digested with five restriction enzymes, four of which (HaeIII, HinfI, Msp/HPaII, and AluI) were capable of digesting the genetic material of these strains, providing small fragments of kDNA (though HaeIII did not digest the kDNA of the amaranth strain).

The sum of the lengths of the fragments obtained was greater than the length of the linearized minicircles–approximately 1.6 kb, coinciding with earlier studies by our team (Muller et al. 1995; Fernández-Becerra et al. 1996). The numerous bands of small size to which the linearized minicircles; indicated high heterogenety in the sequence as previously found in other trypanosomatids (Simpson 1987; Ryan et al. 1988). The minicircles of the three strains were highly heterogeneous, as were those previously obtained using strains isolated from tomato and cherimoya fruits.

The restriction patterns revealed the presence of very faint bands corresponding to molecules of high molecular weight, these probably being broken maxicircles, which were detectable only when great quantities of DNA were deposited in the gel.

As revealed in the studies of kDNA, the similarity in the electrophoretic profiles of the strains isolated from mango and clover strengthen the contention discussed above in relation to isoenzyme studies of variability–that is, that these two isolates constitute one group of trypanosomatids from plants phylogenetically very close to one another and distant from the strain isolated from amaranth. In addition, the strong similarity of these electrophoretic profiles, and of those previously obtained from tomato and cherimoya fruits (Fernández-Becerra et al. 1996), as well as their geographic proximity, confirm that this is one strain capable of parasitizing different plants and that some of these plants (clover, for instance) can even act as reservoirs for the parasites. In addition, the possibility remains that an insect could act as an intermediary host, feeding on the different plants, or that the infection occurs without the need for completion of a biological cycle in an insect and that these insects act as vectors to transmit the disease from one plant to another (Camargo et al. 1990; Muller et al. 1995).

Phytomonas depends totally on glycolysis to obtain energy. The special compartmentalization that these organisms show in the glycolytic pathway enable them to direct this process more efficiently than would a conventional eukaryotic cell (Sánchez-Moreno et al. 1992). This capacity to use sugars rapidly and efficiently is highly valuable for adaptation to and colonization of different hosts, particularly given that these organisms lack a normal system of carbohydrate storage (Vickerman 1994). Incapable of completely degrading glucose to CO_2 under aerobic conditions (Sánchez-Moreno et al. 1992), *Phytomonas* spp. excrete a great part of their carbon skeleton into the medium as fermentative metabolites, the proportion depending on the species (Blum 1993). *Phytomonas* consumes glucose at a great rate, thereby acidifying the culture medium due to incomplete oxidation to acids (data not shown).

To identify the acids produced and excreted into the culture medium by the three strains isolated, we used [^{1}H]-NMR spectroscopy, a technique used in the last few years to study the metabolism of parasitic protozoa (Thompson 1991). The metabolites excreted by these parasites were acetate, ethanol, glycine, glycerol, and succinate. No qualitative difference was found between the three isolates, although we did find quantitative differences in the production of acetate and ethanol in the strains of amaranth with respect to those of mango and clover.

The catabolites excreted by the three strains did not differ from those produced and excreted by other *Phytomonas* species (Sánchez-Moreno et al. 1992, 1995b; Chaumont et al. 1994); this suggests that the catabolism of *Phytomonas* in culture follows a single pattern in all species or isolates.

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