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MORPHOLOGICAL AND ULTRASTRUCTURAL FEATURES OF A STRAIN OF *BOTRYOCOCCUS TERRIBILIS* (TREBOUXIOPHYCEAE) FROM BRAZIL[1]

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The genus *Botryococcus* comprises a group of cosmopolitan species of freshwater colonial green algae, some of which synthesize and accumulate an unusually high level (15–76%) of liquid hydrocarbons. This characteristic suggests the possibility of exploiting species from this group as renewable sources for jet fuel. An oil-rich strain of *Botryococcus* (Trebouxiophyceae) was isolated from a freshwater pond in the state of Bahia, Brazil, and is presently maintained under standard conditions at the Culture Collection of the Institute of Biology, Federal University of Bahia. The taxonomic classification of the species was based on light microscopy (LM); and TEM and SEM were used to better characterize its features, which have never before been described at this level. The LM characterization included the size of the colonies (35.7–157 μm) and cells (8–10 × 5–9 μm) and their connection in sub-colonies by mucilaginous strands, as well as the presence of mucilaginous processes on the periphery of some of the colonies, with most of the cells included inside the colony. Reproduction occurred through divisions into two to four autospores. These features characterized the species as *Botryococcus terribilis* Komárek and Marvan. The TEM study showed, in addition to the presence of starch grains, pyrenoids that are penetrated by thick thylakoids. The pyrenoid bodies appear as electron-dense protein inclusions located in the chloroplast and surrounded by a starch sheath. These structures, which contain most if not all of the Ribulose-1,5-bisphosphate carboxylase oxygenase in several algal species that have been studied closely, are newly discovered for this species.

Key index words: **biofuels; *Botryococcus terribilis*; morphology; taxonomy; ultrastructure**

Botryococcus Kützing is a group of planktonic green algae that is often cited in reports on floristic taxonomy, limnology, and biotechnology. Members of the group are widely distributed in fresh- and brackish-water, oligo- to mesotrophic lakes, reservoirs and ponds around the world, where they can grow massively (Metzger and Largeau 1999, Volova et al. 2003). These species, especially *Botryococcus braunii* Kützing, have attracted attention since they produce lipids, lipid esters, and liquid hydrocarbons (Li and Qin 2005). All these substances are usable for biofuel production, although their chemical nature may vary with the producer strain (Banerjee et al. 2002). For *B. braunii,* three different biochemical races have been documented, according to the characteristic hydrocarbon that they produce (Wake and Hillen 1981, Metzger and Largeau 2005, Mata et al. 2010). The A race produces *n*-alkadienes and trienes, while the B race produces triterpenoid

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hydrocarbons (botryoccocenes), and the L race produces lycopadiene, a C_{40} tetraterpene hydrocarbon (Banerjee et al. 2002). The A and B races are distributed worldwide, while the L race is so far known only from the tropics (Metzger and Largeau 2005).

Under light microscopy examination, the group, in general, exhibits a morphology characterized by a colonial organization of individual cells, which are held together by a refringent gelatinous matrix containing lipids that are easily excreted by pressure. The hydrocarbons are stored in the matrix surrounding the cells. They consist of outer walls originating from successive cellular divisions (Metzger and Largeau 2005). However, in view of the morphological heterogeneity of the size and shape of the cells and colonies, related to the physiological characteristics of the strains, aspects of the taxonomy of the group are still under debate.

The genus *Botryococcus* was included in the family Botryococcaceae Wille 1909, order Chlorococcales (Komárek and Fott 1983). According to ultrastructural and molecular phylogenetic studies (Pickett-Heaps and Marchant 1972, Melkonian 1980, 1990, Friedl 1995, 1997), the order Chlorococcales *sensu lato* represents a mixture of organisms belonging to different lineages, generally distributed in two main classes of unicellular green algae, the Chlorophyceae and the Trebouxiophyceae.

No comprehensive molecular study has yet treated the family Botryococcaceae as a whole. Only the genus *Dictyosphaerium* Näg., formerly included in this family, is related to the *Parachlorella* clade in the family Chlorellaceae (Trebouxiophyceae) (Krienitz et al. 2010, Bock et al. 2011).

Several taxonomic contributions published since 1990 include those of Hindák (1991), Komárková (1991), Komárek and Marvan (1992), Comas and Pérez Baliero (2002), Koreivienė and Kasperoviciene (2003) and Fanés Treviño et al. (2009). However, the publication of Komárek and Marvan (1992) is the only world monograph of the genus. These authors studied natural populations collected from different geographical regions, and recognized 13 different morphotypes. Considering that Plain et al. (1993) noted that for the same strain, some features vary in relation to age and culture conditions, nine of the 13 species characterized by Komárek and Marvan (1992) are properly accepted as species, and the taxonomic categories of four morphotypes are not defined.

The identification of the genus remains particularly difficult, since the diagnostic characters (colony shape, partial or total immersion of the cells within the colonial matrix, cell shapes, etc.) are highly variable, and the dense matrix of colonial mucosa within the cell, when viewed under the light microscope, often makes it difficult or impossible to observe the pyrenoids or pyrenoid bodies, inclusions, etc.

The presence of pyrenoids is an enigmatic problem in the genus *Botryococcus*. When the pyrenoid is small, the type cannot be determined under the light microscope, and the use of TEM is necessary (Komárek and Marvan 1992).

Several investigators have reported members of the genus *Botryococcus* from Brazil. Rodrigues et al. (2010) recorded *Botryococcus neglectus* (West and G. S. West) Komárek and Marvan and *Botryococcus terribilis* Komárek and Marvan in two reservoirs in São Paulo, state of São Paulo; and Hentschke and Torgan (2010) reported *B. braunii* from different aquatic environments on the coastal plain of Rio Grande do Sul. Nogueira and Oliveira (2009) reported, in addition to *B. terribilis*, the occurrence of *B. braunii* in lakes in Goiânia, state of Goiás; and Santos et al. (2009) collected species of the genus from five Brazilian hydroelectric reservoirs, although they did not provide identifications at the species level. Carvalho (2003), in a doctoral study on biomonitoring in reservoirs in São Paulo, state of São Paulo, found *B. braunii*, *Botryococcus protuberans* West and G. S. West, *B. terribilis,* and one species that was not identified to species level. In Lake Batata, Pará, *Botryococcus fernandoi* J. Komárek and P. Marvan was recorded by Huszar and Reynolds (1997).

Botryococcus terribilis described by Komárek and Marvan (1992) is one of the most frequent species found in Cuba (Comas 1996) and southern Spain (Fanés Treviño et al. 2009). The climate characteristics in the regions where this species has been found indicate a tropical distribution.

Of all the *Botryococcus* species, ultrastructural studies have been conducted only for *B. braunii* (Schnepf and Koch 1978, Wolf and Cox 1981, Beakes and Cleary 1999, Noguchi and Kakami 1999). This relatively small number of studies contrasts with the importance of this taxonomic group as the only one reported to produce an unusually rich renewable source of hydrocarbons, which can be converted to gasoline and jet fuel. To partly fill this gap, this article describes characteristics of *B. terribilis* that are novel or differ from those described in earlier studies on this species. We attempt to relate some ultrastructural features of *B. terribilis* with its lipid-producing character, thus showing the potential of this species as a source of biofuel.

MATERIAL AND METHODS

Collection. Following the protocol for the establishment of the Native Algae Collection at the Laboratory of Marine Biology and Biomonitoring (LABIOMAR), Institute of Biology (IB), Federal University of Bahia (UFBA), samples were collected from the Caixão Lagoon (12°93′26.38″S – 38°39′09.41″W), Bahia, Brazil, using a system developed at the laboratory. This system, which can retain microalgae <100 μm, consists of a piece of PVC (polyvinyl chloride) with three outputs, one of which is closed to store the concentrated sample. On the lateral output, a plankton net (20 μm) is attached to retain organisms between 20 and 100 μm, and smaller organisms after the net becomes clogged. The third output is fitted with another plankton net (100 μm) and a long PVC tube in order to filter out larger predators.

Algal isolation and culture. Fractions of natural samples were enriched with modified Chu 13 medium (Daynanda et al. 2006). The target species was isolated by the usual techniques (Andersen and Kawachi 2005, Lourenço 2006) to obtain monoalgal cultures. The strains were maintained in sterile flat-bottomed polystyrene plates with lids, until the completion of the adjustment phase, and were then transferred to test tubes with Chu 13 medium and kept in a growth chamber at 25°C, under 24 h illumination (114 $\mu E \cdot m^2 \cdot s^{-1}$). The strains received number code FW-15. To obtain samples in the exponential growth phase, the colonies were cultured in Erlenmeyer flasks with CO_2 (2%) and constant agitation (80 beats per minute), under a 12:12 h photoperiod.

Light microscopy – LM. Light microscopy was carried out using an Olympus BX-51 microscope (Olympus, Tokyo, Japan). Digital images were captured with an Olympus Q-Color5™ camera, using the software Image-Pro® Plus 6.2 (Media Cybernetics, Bethesda, MD, USA).

Transmission electron microscopy – TEM. The ultrastructural studies were performed according to the protocol used by the Electron Microscopy Facility of the Gonçalo Moniz Research Center, Oswaldo Cruz Foundation. Colonies were fixed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer for 24 h at 4°C. The colonies were washed in the same buffer and postfixed with 1% OsO_4 at room temperature (1 h), and some samples with 0.8% potassium ferricyanide/5 mM $CaCl_2$ and Alcian Blue. The material was rinsed in buffer, dehydrated through a graded acetone series, and embedded in PolyBed® resin (Polyscience, Inc., Warrington, PA, USA). Thin sections were cut with a diamond knife on a Reichert Supernova ultramicrotome, stained with uranyl acetate and lead citrate, and observed under a Zeiss EM109 transmission electron microscope (Zeiss, Oberkochen, Germany). The images were acquired using the Mega View camera (Soft Imaging System GmbH, Müenster, Germany).

Scanning electron microscopy – SEM. The colonies were fixed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer for 24 h (4°C). The colonies were washed in the same buffer, postfixed with 1% OsO_4, adhered to coverslips, dehydrated in an ethanol series, critical point-dried in a Leica EM CPD030 apparatus, and mounted on specimen stubs. The samples were ion sputtered with a 2–3 nm gold layer and observed using a JEOL JSM 6390LV SEM (JEOL Ltd., Tokyo, Japan).

In the present study, we follow Komárek and Fott (1983) in including *Botryococcus* in the family Botryococcaceae, but consider it to be a member of the Trebouxiophyceae, because the molecular data support the placement of *B. braunii*, the type species for the genus and family, in this group (Sawayama et al. 1995, Okada et al. 2000, Senousy et al. 2004, Kagiwada et al. 2005, Weiss et al. 2010).

RESULTS

Characterization by light microscopy. The colonies were irregularly spheroid or irregularly ellipsoid (35.7–157 μm diameter), sometimes consisting of subcolonies joined by gelatinous strands (Fig. 1a). Colonies were green or orange to brownish, firm, homogeneous or with indistinguishable lamellate margins that usually completely covered the cell groups. Inside the colony matrix may appear cell-wall-bound vesicles, full of lipid droplets that are released at the colony surface or through elongated surface processes.

The margins of the colony may have mucilaginous processes of 3.17–12.8 μm in length, simple or branched (Fig. 1b). In the strain studied herein, some colonies showed these processes after collection, but after continuous culture either the processes disappeared or only a few colonies were able to maintain them.

Cells. Basically obovoid in lateral view, rounded in polar view, mostly fully covered by the colony envelope but with their apical portions sometimes emerging. Dimensions: 8–10 × 5–9 μm (Fig. 2).

Chloroplastids. Parietal, lateral or apical, never basal with one to five refractive pyrenoid-like bodies (Fig. 2).

Pyrenoids. According to our observations, within the chloroplastid can be seen refractive pyrenoid-like bodies, the nature of which has not yet been chemically determined (Fig. 2).

Reproduction. The cells propagate by formation of 2-(4) autospores, with their longitudinal axis more or less in parallel, arranged within the mother cell (Fig. 2). The features of the microalgae analyzed agree in general aspects with the original description and iconotypus of the species *B. terribilis* (Komárek and Marvan 1992, pp. 92, 94, 97, figs. 23–24).

SEM observations. All the colonies had a dense appearance (Fig. 3), with cells closely crowded and subcolonies scattered or grouped, with conspicuous mucilage extending between them (Fig. 3a). Figure 3b shows the colonies without residues of

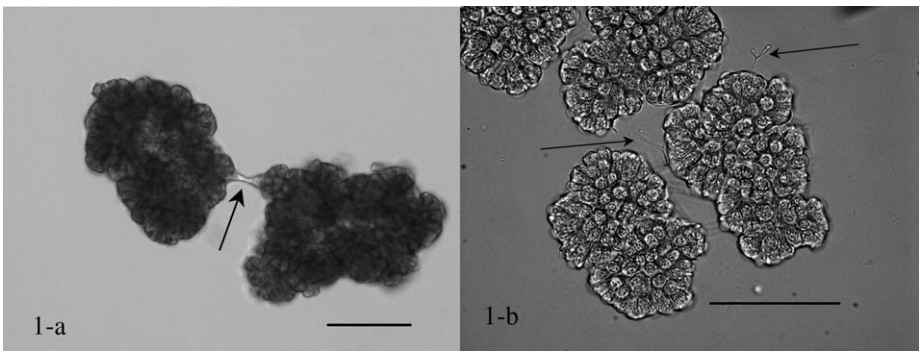

FIG. 1. Light micrograph of *Botryococcus terribilis*. (a) showing subcolonies connected by gelatinous strands (arrow); (b) detail of mucilage process (arrows). Scale bars 50 μm.

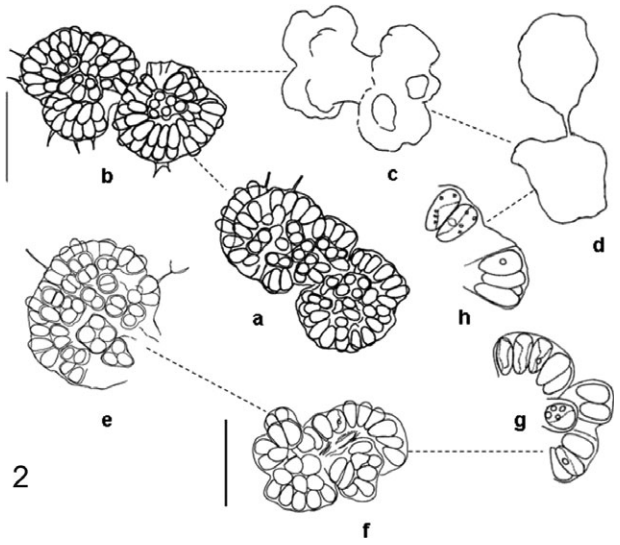

FIG. 2. Scheme of *Botryococcus terribilis*: (a–d) general appearance of colonies; (e–h) cells with chloroplastids and pyrenoid-like bodies. Scale bars 50 μm.

mucilage, and the autospores (2–4). To explain why mucilage may be present or absent, additional studies are needed.

TEM observations. Cells (Figs. 4 and 5) were collected without treatment or a specific growth phase, and fixed with additional potassium ferricyanide. The cup-shaped chloroplastid consists of two lobes that are subdivided into smaller segments to the cell apex. TEM clearly showed pyrenoids, located laterally or somewhat toward the basal end, usually surrounded by elongate starch granules; but were sometimes naked and therefore undetectable by means of light microscopy. These pyrenoids may be penetrated by 1–2 relatively thick thylakoids, which mostly pass completely through them. Along with pyrenoids, one to several starch grains may occur within the plastid (Fig. 4). The formation of autospores is shown in Fig. 5, with a new cell wall, but with the mother-cell wall still persisting.

In Fig. 6, is shown the colonies that were collected in the initial growth phase, without potassium ferricyanide in the fixation process. The cell apex shows a clear region and a chloroplastid with dense thylakoids (6 a/b). In Fig. 6c, the hydrocarbon-containing colony matrix appears even and dense. Also, many lipid droplets are present between the plasma membrane and the cell wall.

The cells in Fig. 7 were obtained without selecting the culture stage, and were stained with additional Alcian Blue to enhance the membrane. Unknown material can be seen exuding from the apical cell (Fig. 7a). Several mitochondria and plastoglobules within chloroplastids can be observed in Fig. 7b.

Description: *Botryococcus terribilis* Komárek et Marvan

Arch. Protistenkd. 141: 92, 94, 97, figs. 23–24. 1992.

Colonies irregularly spherical, irregularly ellipsoidal to more or less elongated in one direction, 35–157 μm in diameter, formed by partial groups of more or less radially oriented cells, which are

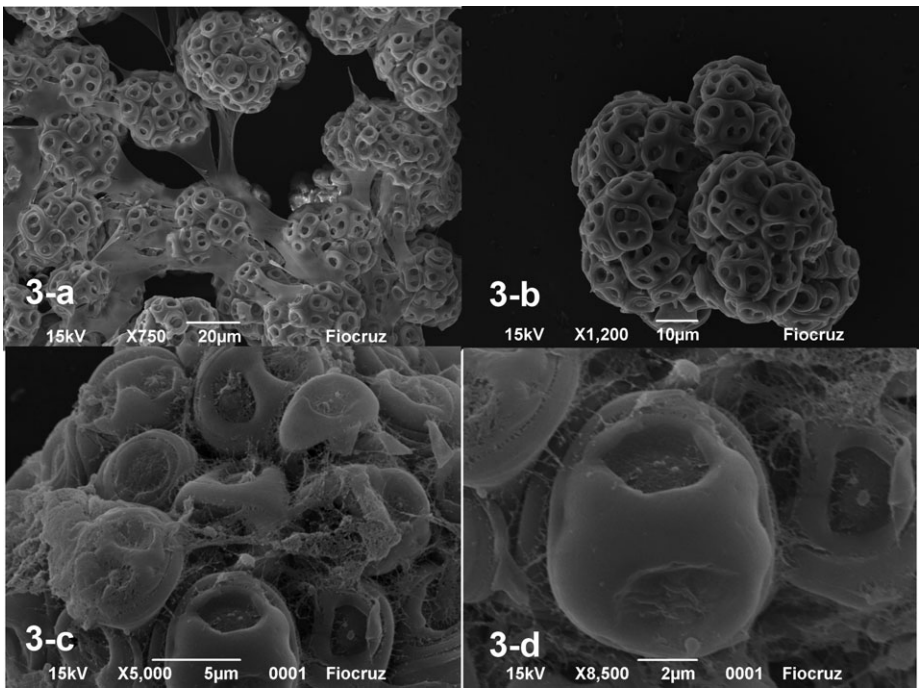

FIG. 3. Scanning electron microscopy of *Botryococcus terribilis*. (a and b) show the protocol without utilization of the critical point, versus (c and d) with this traditional protocol. (a) residual mucilage covering a group of cells, probably from the colonial matrix. (d) high-magnification image of (c), showing detail of cells and fragmented mucilage in the form of a residual network.

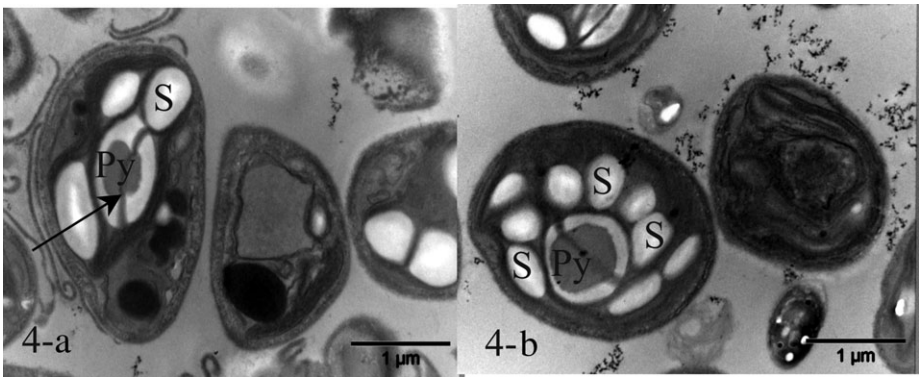

FIG. 4. Transmission electron microscopy of *Botryococcus terribilis*. (a) cell in longitudinal section, showing pyrenoid, starch, and chloroplastid with thylakoids (arrow) crossing pyrenoid. (b) cell in cross section, with many starch granules and a large pyrenoid. Abbreviations: Py-pyrenoid, S-starch, Ch-chloroplastid.

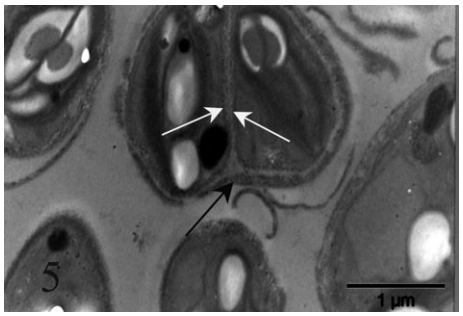

FIG. 5. Transmission electron microscopy of *Botryococcus terribilis*. Cell wall formation of autospores, with daughter-cell wall (white arrows) and remaining mother-cell wall (black arrow).

usually completely hidden within colonial mucilage, sometimes emerging by their apical parts. Colonies sometimes composed by sub-colonies joined by mucilaginous strands. Mucilage firm, colorless, green, orange or brownish, homogeneous or irregularly layered. Inside the colonies sometimes hollow vesicles surrounded by firm walls. Colonial margins sometimes covered by simple or divaricated gelatinous processes, 3.17–12.8 μm long, sometimes lacking; processes often with terminal oil drops. Cells 8–10 × 5–9 μm, in lateral view basically obovoid, more or less rounded in polar view, without mucilaginous apical caps; cells usually with a single parietal chloroplastid, lateral or apical (never basal), with a naked pyrenoid or covered by starch, usually crossed by 1–2 thick thylakoids, very often together, pyrenoids one to few pyrenoid-like bodies (intraplastidial starch grains). Reproduction by 2–4 autospores arranged in parallel in the mother cell.

DISCUSSION

In natural populations, colonies of *B. terribilis* reach ~100 μm in diameter (Komárek and Marvan 1992, Fanés Treviño et al. 2009). According to our observations, if reared under low illumination, the colonies may exceed these dimensions.

The full inclusion of cells within the colony is a distinctive characteristic of the species *B. terribilis*, according to Komárek and Marvan (1992); however, in the Brazilian and Cuban populations observed by A.A. Comas (unpublished data) and in southern Spain (Fanés Treviño et al. 2009), very often the colonies have the apical parts of their cells emerging to 1/3 of the cell length. However, in most colonies the cells are covered by the colonial matrix. Perhaps this is a variable character. In *B. braunii* also, some cells are embedded within the colonies, while most of them emerge from the colonies by 1/3–2/3 of the cell length (Fanés Treviño et al. 2009). They did not observe apical mucilaginous caps covering the distal ends in the cells of *B. braunii*.

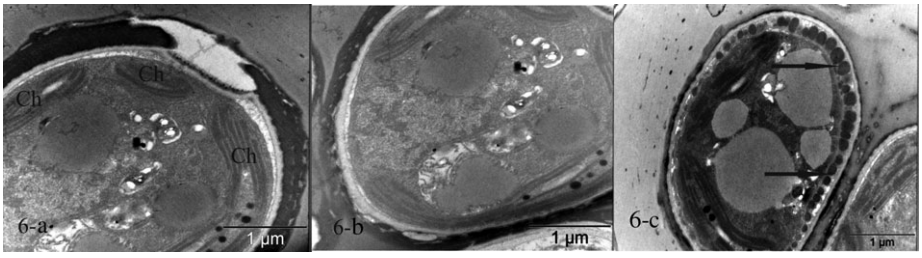

FIG. 6. Transmission electron microscopy of *Botryococcus terribilis*. (a) longitudinal section showing one chloroplastid on the side, closet to the plasma membrane; on the other side of the cell, two segments of the chloroplastids can be seen, also located very close to the plasma membrane. (b) detail of cell end. (c) many lipid droplets between plasma membrane and cell wall (white arrow).

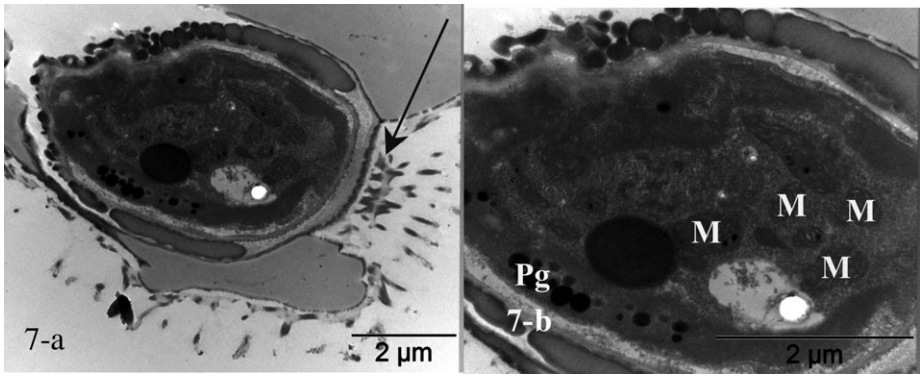

FIG. 7. Transmission electron microscopy of *Botryococcus terribilis*: (a) cell showing exudation process on the cell apex (arrow). (b) high-magnification image of (a), showing several mitochondria and large plastoglobules. Abbreviations: M- mitochondria, Pg-plastoglobules.

Although this is a variable character (Koreivienė and Kasperoviciene 2003, Fanés Treviño et al. 2009), it is a good point of distinction between the two species, at least in the concept accepted by Komárek and Marvan (1992), represented by the strain DROOP 1950/807 from the Göttingen Collection.

The presence and dimensions of the processes are variable (Fanés Treviño et al. 2009) and may possibly be influenced by environmental conditions (Komárek and Marvan 1992). We collected colonies both with and without processes. Few of the colonies that initially had processes were able to maintain them in continuous culture. Plain et al. (1993) also observed that in the same strain of *B. braunii*, some features varied according to age and culture conditions.

The observation of pyrenoids in cells of *B. terribilis* is the first confirmation of their presence in this species. In general, the presence of pyrenoids in *Botryococcus* has been controversial, since with light micrography this organelle and other inclusions are difficult to observe, especially in natural populations. Komárek and Marvan (1992) noted the lack of pyrenoids in *B. terribilis*, and indicated only the presence of pyrenoid-resembling inclusions or starch granules. The presence or absence of pyrenoids was one of the features used by Komárek and Marvan (1992) to separate the various *Botryococcus* populations in accordance with the generally used concept. This structure contains most if not all of the ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) in several algal species that have been studied closely (Badger and Price 1994).

Schnepf and Koch (1978) demonstrated in *B. braunii* (strain DROOP 1950/807) the presence of a naked pyrenoidal body (without starch sheath), but surrounded by several starch granules. In the same species, Wolf and Cox (1981) found undefined pyrenoidal bodies penetrated by one or a group of thylakoids at more or less regular intervals, with starch grains in close proximity (not forming a sheath). According to the latter authors, the density of thylakoids is greater within active-state chloroplasts than in resting-state ones. The thylakoids shown in our studies (Fig. 6, a and b) correspond to chloroplastids in the active state, confirming the statement of Wolf and Cox.

The other feature of *B. terribilis* that was similar to *B. braunii* was the presence of an even and dense colonial matrix, as also described by Wolf and Cox (1981) for *B. braunii*.

The formation of autospores was similar to that found by Wolf and Cox (1981) and Noguchi and Kakami (1999) in a strain of *B. braunii*, with the production of a new cell wall, but with the mother-cell wall still persisting.

Botryococcus terribilis, described by Komárek and Marvan (1992) from the reservoir ''Zemplínská šírava (Czech Republic) cited by these authors, in addition to Austria, Chad, Cuba, New Zealand, and southern Sweden, has, therefore, a wide distribution in the plankton of alkaline reservoirs. One of the most common taxa in Brazil (Carvalho 2003, Nogueira and Oliveira 2009, Rodrigues et al. 2010, A.A. Comas, unpubl.,) and southern Spain (Fanés Treviño et al. 2009), apparently it is more common in warmer areas of the planet.

Botryococcus terribilis has been frequently misidentified as *B. braunii*, but as Komárek and Marvan (1992) stated for *B. terribilis*, is ''distinguished by its cells usually within the colonial matrix (exceptionally only their apical portions protruding, without apical mucilaginous caps), the surface of the colony sometimes covered by elongated mucous processes, simple or irregularly branched and their cells with a lateral or apical chloroplastid (not basal)''. However, the presence of pyrenoids has been confirmed, as well as intraplastidial starch grains (pyrenoid-like bodies). Another differentiating character is perhaps the cellular dimensions in relation to the size of the colonies. According to Fanés Treviño et al. (2009), *B. terribilis* has larger cells in relation to the colony diameter in comparison with *B. braunii*, where the cells are smaller relative to the colony diameter, and therefore, the latter has a comparatively larger number of cells.

Without a doubt, in our observation, the differences between the two species (*B. terribilis* and *B. braunii*) are striking, especially including the concentration of cells in the colony, with cells of *B. terribilis* densely packed and lacking apical mucilaginous ''caps'', while *B. braunii* (our personal observation of a strain from UTEX maintained in the Culture Collection of LABIOMAR/IB/UFBA) has loose clusters of cells and the presence of mucilaginous ''caps''.

Another species related to *B. terribilis* is *B. neglectus*. This species (based on *Ineffigiata neglecta* W. et G. S. West 1897 from Ireland) also forms mucilaginous processes on the colony margins. Marginal mucilaginous processes were also found in *Botryococcus australis* Komárek et Marvan (Comas and Pérez Baliero 2002). According to the original diagnosis of *B. neglectus* (Komárek and Marvan 1992), the alga has colonies and cells with smaller dimensions, as well as a particular ecology and distribution: from oligo- to mesotrophic waters in temperate regions of the Northern Hemisphere (Fanés Treviño et al. 2009); whereas *B. australis* has larger colonies and narrow cells (15–19 × 4–7 μm) (Komárek and Marvan 1992).

Rodrigues et al. (2010) also found relatively small colonies and cells (4.5–5.0 × 2.5–3.0 μm) in *B. neglectus*. In our results, *B. terribilis* showed cells with intermediate dimensions (8–10 × 5–9 μm). We conclude that the size of cells can be used as an additional feature for distinguishing among *Botryococcus* species.

In our strain, the unknown material exuding from the apical cell was similar to that found by Berkaloff et al. (1984) in their studies with a strain of *B. braunii,* and they considered the material to be concentrated polysaccharide fibers.

The presence of several mitochondria and plastoglobules within chloroplastids generated a controversy. According to Wolf and Cox (1981), the presence of several mitochondria indicates that the cell may be in the exponential phase, while the presence of large numbers of plastoglobules is generally related to resting cells. In our case this is contradictory, because the ultrastructure of the cell has a peculiar way of combining structures of different growth phases. New studies are being conducted to clarify this issue, with samples obtained in the different growth phases.

Considering the data presented herein, such as the presence of a pyrenoid, thylakoids, the observation and position of organelles (mitochondria and chloroplasts), formation of autospores, plastoglobules, exudation of polysaccharide fibers, and lipid droplets showed that the use of TEM is an important tool in the ultrastructural characterization for the species of *Botryococcus,* particularly *B. terribilis*. These data, together with morphological features observed by light microscopy, provide additional insights for the characterization of the species.

The present article reports the first TEM and SEM observations to characterize the species *B. terribilis,* thus contributing to knowledge of some ultrastructural and cytological aspects of another species of the genus *Botryococcus*.

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