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Botryococcus braunii var. *showa* (Chlorophyceae) from Berkeley, California, United States of America

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Botryococcus braunii var. *showa* has the largest cells and colonies of the genus. Rapidly growing colonies are typically small and shaped like peanut pods, but slowly growing colonies form large irregular aggregates. Only vegetative reproduction has been observed. This new variety is distinguished by production during growth of botryococcene hydrocarbons which comprise up to a third of its dry weight. A 32-carbon botryococcenoid which bears a cyclohexyl moiety occurs only in this variety.

Key Index Words: Axenic culture—botryococcene—hydrocarbons—taxonomy—variety of Botryococcus braunii.

Of seven described species of the genus, *Botryococcus braunii* Kützing has been the only one used for physiological investigations. With rare exceptions, observations have been based on strains with origins from a single common isolate (BERKALOFF and ROSSEAU 1984; WOLF *et al.* 1985) deposited in 1950 by Droop at the Center for Culture of Algae and Protozoa (CCAP; ASHER and SPALDING 1982). Investigations on the CCAP isolate and its clones consistently showed slow growth rates and low hydrocarbon production (CHIRAC *et al.* 1986) as compared with field observations (WAKE and HILLEN 1980; 1981) of blooms with high lipid contents, an ambiguity that has been a source of concern to those seeking renewable sources of hydrocarbons from algae (WOLF 1986; HILLEN *et al.* 1980). A resting phase in the life history was considered to be necessary for accumulations of large quantities of hydrocarbons (BROWN *et al.* 1969), since it could be used to trigger accumulation of linear hydrocarbons in the CCAP isolate and branched hydrocarbons in some other isolates (BERKALOFF and ROUSSEAU 1984; AARONSON and PATNI 1986). Different from all other reported isolates, the present new variety, previously referred to as the Berkeley strain (OHMORI *et al.* 1985; WOLF *et al.* 1985a; WOLF *et al.* 1985b), shows rapid metabolism of carbon into branched-molecule hydrocarbons during active growth (WOLF *et al.* 1985b) and is morphologically and chemotaxonomically distinct from other *B. braunii*.

Materials and Methods

This new variety was originally isolated in June 1980 from culturing tanks for flowering aquatic *Menyanthes* plants located in a greenhouse on the fifth floor of the Life Sciences Building of the University of California, Berkeley, CA 94720. The aquatic plants were from a tropical African collection made by R. Ornduff, maintained with tapwater at ambient room temperature (15–30°C).

Whole soil-water extract (ASHER and SPALDING 1982) was steam sterilized and

buffered with phosphate at pH 7. The soil extract was supplemented with Provasoli's enrichment solution at 0.25 normal strength with an equal volume of 1-μm-sterile-filtered lily-culture water. Two thousand sterile disposable test tubes (12 × 75 mm) with friction-fit clear plastic caps were each given 1.5 ml of the medium.

Floating orange colonies were collected from the water in the plant culturing tank, and the suspension was diluted with an equal volume of sterile enriched medium. Single colonies were selected by micropipette. Single orange colonies selected by means of an inverted microscope at low power (40 ×) magnification were transferred to test tubes containing enrichment medium.

The test tubes were enclosed in plastic bags which were deflated and then filled once with carbon dioxide:nitrogen gas 1:1. The cultures were maintained under 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ cool white fluorescent illumination at 30°C. Cultures were continuously illuminated. When numerous colonies were observed in the tubes, additional medium was added. Within two weeks, cultures that were contaminated were removed. Approximately 200 unialgal cultures resulted from the 2000 single colony isolates. One culture that was tested contained high levels of botryococcenes. This culture was originally called the Berkeley strain is the present new variety.

Maintenance of the new isolate is undertaken in Bold's basal medium (ASHER and SPALDING 1982) without NaCl. The pH was maintained by providing a cloak of carbon dioxide since slow growth was observed when Tris buffers were added.

Axenic cultures were isolated by gentle homogenization of the colonies to shake off contaminants. Colonies were washed with several volumes of sterile medium. Remaining colonies were resuspended in sterile water and spread on agar plates with nutrient medium for growth. Clean colonies were transferred to yeast extract agar plates to test for presence of bacterial contamination. Final colonies were placed in aqueous media as above, but with the addition of 0.1 mM sucrose and 10 mM HEPES buffer to replace the carbon dioxide.

Diagnoses and Observations

Botryococcus braunii var. *showa* Nonomura var. nov.

Chlorococcales, Chlorophyta

Coloniae huius varietatis virides, flavae, aurantiacae aut brunae secundum regimen lucis aut statum physiologicum culturae. Coloniae ad superficiem culturarum quietarum fluitant. Coloniae forma magnitudine a 25 ad 300 μm variant, et ex una ad aliquot aggregationes cellularum irregulares ad sphericas constituant. Coloniae cellularum matrice hydrocarbones abundantes continente cohaerent. Coloniae rapide crescentes forma leguminis *Arachis hypogaeae*, e duarum submonadum fere sphericarum constitutae. In colonis tarde crescentibus in culturis veteribus, aggregationes coloniarum multiplicarum forma irregularum vulge repertae.

Cellulae constitutentes colonias 10–30 μm long., forma ovoideae, cuneatae, pyriformes aut irregulares. Cellulae in poculis matricalibus, quae elementa membranae cellularis atque strata hydrocarbonis continent. Deposita hydrocarbonis intracellulariter et in matrice et in superficie colonarium adsunt. Membrana cellulae crassa, e strato interiore polysaccharidi et e structura exteriore tenui trilamellari constituta. Cellulae nucleum, unum dictiosoma anterius atque chloroplastum poculiformem, pyrenoide basali praeditum, continent. Organella insolita nulla. Deposita hydrocarbones 0.5 ad 20 μm diam. in cytoplasmate et in membrana et matrice adsunt. Reproductio tantummodo fissione binaria effecta. Post cytokinesem poculum novum matricale circum omnem cellulam formatur. Fragmentio propagationem coloniarum efficit.

Coloniae in aqua dulci ad quasi subsalsam nutrimento locupletam sub luce continua aut periodica (e.g. 16:8 h LD) crescunt.

Coloniae rigores lucis magnos tolerant (e.g. 150–250 $\mu E \cdot m^{-2} \cdot sec^{-1}$).

Varietas nova a *Botryococcus braunii* typica (e.g. isolata e Collectione Culturarum in loco Cambridge dicto) differt structura coloniae atque biochima. Coloniae in *B. braunii* typica plus minusve planae, et e cellulis radiatim ordinatis unico in strato periferam versus conpositae, quandoquidem coloniae varietatis novae globosae, cellulis in stratis multiplis ordinatis. Varietas nova plures hydrocarbones botryococcinas (30% vel plus ponderis secci), quam *B. braunii* typica 1.5–20% producit. Hydrocarbones a varietate nova producti insignes, praebentes illam $C_{32}H_{54}$ compositionem C_6 anulo terminali praeditam (MURAKAMI *et al.* 1988). Physiologice, varietas nova ab aliis gentibus metabolismo rapido carbonis nutrientis in hydrocarbones tempore crescentiae activae insignita.

Locus typi: Cisternae pro culturea *Menyanthes* in caldaris ad contignationem quintam sedificationis pro scientiis vitalibus Universitatis Californicae locum Berkeley, CA 94720, United States of America dictum.

Epitheton huius varietatis e "Showa" e litteris Japonicis, 昭和, "Pacem nitentem" significante derivatum.

Axenic and unialgal cultures are morphologically identical. The alga is colonial and floats at the surface of still cultures. Colonies range from 25–300 μm and consist of one to several irregular to spherical aggregates of cells. The colonies are held together by a matrix that is rich in hydrocarbons. Rapidly growing colonies are shaped like peanut pods (Fig. 1), being composed of two approximately hemispherical subunits approximately 25–50 μm long. In slowly growing colonies or in old cultures, large multiple-colony aggregates of irregular shape are common.

Cells composing the colonies are 10–30 μm long and are ovoid, cuneate, pyriform or irregular in shape. They are individually embedded in matrix cups that comprise cell wall components and layers of hydrocarbon. Hydrocarbon deposits are present intracellularly, in the matrix and on the surface of colonies. The cell wall is thick, composed of an inner polysaccharide layer and a thin outer trilamellar structure. Cells contain a nucleus, one anteriorly located dictyosome and a cup-shaped chloroplast with a basal pyrenoid. No unusual organelles are present. Deposits of hydrocarbon 0.5 to 2.0 μm in diameter are present in the cytoplasm and in the wall matrix. Binary fission is the only form of reproduction. A new matrix cup is formed around each cell following cytokinesis. Fragmentation causes propagation of the colonies.

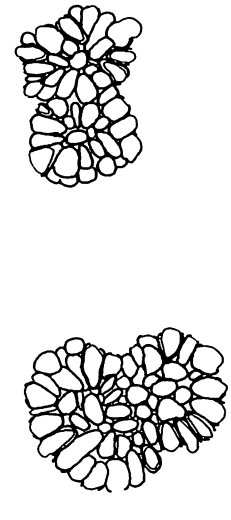

Fig. 1. Camera lucida drawings showing the peanut-pod shape of the colonies of *Botryococcus braunii* var. *showa* and some cell shapes in matrix cups

Colonies grow in nutrient-enriched fresh to slightly brackish water (0.1 M NaCl) under continuous or periodic (e.g. 16:8 h LD) photoperiod. They are tolerant of high light intensities, growing well, for example, under direct sunlight or under fluorescent lamps at 150–250 $\mu E \cdot m^{-2} \cdot s^{-1}$.

The new variety is distinguished by locality, colony structure and biochemistry. Other varieties of the species are flattened and composed of cells that are arranged radiately in a single layer towards the periphery, whereas, those of the new variety

are globular, often with a median constriction, with cells arranged in multiple layers. The new variety produces more botryococcene hydrocarbons, 30% or more of the dry weight, than culture types of *Botryococcus braunii* (e.g. ASHER and SPALDING 1982) which produced less than 5% botryococcenes under identical conditions. A hydrocarbon produced by the new variety is distinctive, showing a $C_{32}H_{54}$ compound with a terminal C_6 ring (MURAKAMI *et al.* 1988). Physiologically, the new variety is distinguished from other strains by its rapid metabolism of nutrient carbon into hydrocarbons during active growth (WOLF *et al.* 1986). Hydrocarbon droplets are often observed surrounding the colonies and are released into the growth medium.

Morphologically, the new variety is distinguished from other strains by large colony and cell size and peanut (*Arachis*) pod shape of the colonies.

This new variety *Showa* is from Japanese, 昭和, meaning brilliant peace, referring to flames that arise from ignition of the biomass.

A dried type specimen is deposited UCB having accession no. UC147504.

Discussion

HIROSE and OGASAWARA (1977) confirmed the position of *B. braunii* in the Chlorophyceae ultrastructurally. *Botryococcus* species (Table 1) have been described according to colony and cell sizes. The largest colonies of previous description are of *B. giganteus* REINSCH (1877) and *B. protuberans* WEST and WEST (1905) at 218–220 μm diam. far smaller than the new variety at 300 μm diam. *Botryococcus calcareus* WEST (1892) is described from limestone pools and was distinguished from other species by loosely packed cells shaped differently, from subglobose to cubical. In *B. protuberans* WEST and WEST (1905), the cells protrude conspicuously beyond the cell sheath. The cells and colonies of *B. protruberans* var. *minor* SMITH (1918) are smaller (6.5×9.5 μm max. cell diam.) than the type species.

Table 1 Extant species and Varieties of *Botryococcus*

Botryococcus braunii KÜTZING 1849
Botryococcus braunii var. *horridus* HANSGIRG 1905
Botryococcus braunii var. *perarmatus* VIRIEUX 1912
Botryococcus braunii var. *showa* var. nov.
Botryodoccus braunii var. *validus* HANSGIRG 1905
Botryococcus calcareus W. WEST 1892
*Botryococcus giganteus*P .F. REINSCH 1877
Botryococcus micromorus W. et G.S. WEST 1897
Botryococcus protuberans W. et G.S. WEST 1905
Botryococcus protruberans var. *minor* G.M. SMITH 1918
Botryococcus protuberans var. *nanyohensis* JAO 1940
Botryococcus pusillus VAN GOOR 1924
Botryococcus terricola KLEBS 1883

Those of *B. protuberans* var. *nanyohensis* JAO (1940) are also smaller (9.0×18 μm max. cell) than the type species, but our strain differs from *B. protruberans* var. *minor* by larger number of cells and dimension of the colony and wider gelatinous envelopes. The cells of *B. giganteus* REINSCH (1877) are larger (27.8 μm long) than previously described species and nearly as large as the new variety. In contrast, the smallest cells are described for *B. micromorus* W. et G.S. WEST (1897) (3–4.5 μm) and *B. pusillus* Van GOOR (1924) (2–2.5 μm). Zoospores were observed in *Botryococcus terricola* KLEBS (1883), but it was not certain that this form was *Botryococcus*. Notably, a species was misplaced: *Botryococcus sudeticus* Lemmerman (1896) is now *Botrysphaerella* SILVA (1970).

Colonies of *Botryococcus* aggregate to form water flowers and are generally described as being composed of more or less spherical aggregates of cells (FRITSCH 1935). Median constriction in colonial morphology was used to distinguish *Botryococcus pusillus* Van GOOR (1924). Other species in which appressed hemispheres are illustrated include *B. protuberans* W. et G.S. WEST (1905) and *B. calcareus* WEST (1892). The peanut shape of basic colonies caused by budding is a characteristic of the new variety. Within the species, the peanut shape is not described for *B. braunii*, although characteristics of the colony are. The colonies

of *B. braunii* var. *perarmatus* VIRIEUX (1912) are simple or split nearly completely across the diameter; colonies are loosely connected by a mucous strand rather than being appressed, the distinguishing characteristic being long mucous strands. Descriptions of *B. braunii* var. *validus* HANSGIRG (1905) and *B. braunii* var. *horridus* HANSGIRG (1905) were distinguished from each other by the latter variety being equipped with colorless spiny processes over the surface of the colony. One variety of *B. braunii* was named, but *B. braunii* var. *minor* (KALMUS *et al.* 1864) lacks a description.

Five *Botryococcus* spp. are fossils (Table 2). Deposits of boghead coals, coorongite, torbanite and balkaschite were attributed to *B. balkachicus* ZALESSKY (1914; 1926) and *B. coorongianus* THIESSEN (1925). These fossil deposits were ascribed to a common algal source by BLACKBURN (1936) and BLACKBURN and TEMPERLEY (1936) upon demonstration of the interconnection between the coals and realization that the alga was common to living examples of *B. braunii*. Other fossil species, including *B. luteus* TRAVERSE (1955), might also be considered conspecific based on similarities of descriptions to *B. braunii*. A Latin diagnosis is lacking for *B. palanaensis* SAH and KAR (1974).

The major physiological distinction of the new variety is rapid metabolism of carbon into botryococcene (WOLF *et al.* 1985), during exponential growth. This is the only reported B-form (branched botryococcene hydrocarbon) isolate of the genus that synthesizes botryococcenes while it is growing. Previous investigations based on other isolates of *B. braunii* (AARONSON and PATNI 1986; BROWN *et al.* 1969; BELCHER 1968; CHIRAC *et al.* 1985) show that hydrocarbons are produced in large quantity when growth has ceased or is substantially reduced; several isolates (BERKALOFF and ROSSEAU 1984) were designated L-form (Linear Hydrocarbon) synthesizers (WOLF *et al.* 1985a). The production of the hydrocarbon fraction identifiable to the new variety (MURAKAMI *et al.* 1988) was observed in the earliest unialgal isolates and five years later in axenic cultures. The stability of algal strains and the chemotaxonomic value of lipids is supported by HOWARD *et al.* (1983) where chemistries in algae were observed to remain the same under various field and culture conditions. This variety has secretory features that may be exploited for production of hydrocarbons by means of immobilized systems.

Table 2 Fossil Species of *Botryococcus*

Botryococcus braunii KÜTZING 1849
Botryococcus balkachicus ZALESSKY 1914
Botryococcus coorongianus THIESSEN 1929
Botryococcus luteus TRAVERSE 1955
Botryococcus palanaensis SAH et KAR 1974

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The varietal description was translated into Latin by Dr. Hannah CROASDALE. Much of the literature that was over 75 years old was retrieved by Dr. Richard L. MOE, Herbarium, University of California, Berkeley. Dr. MOE also contributed to the manuscript as he and Dr. John A. WEST reviewed the article. The alga was isolated while AMN was a fellow with Dr. Julius SCHACHTER in the School of Medicine, Department of Laboratory Medicine, University of California, San Francisco. Dr. Ralph A. LEWIN, Scripps Institution of Oceanography, University of California, San Diego, made axenic isolates. A patent has been allowed for the new variety by the United States Patent and Trademark Office, Washington, D.C. (Plant 6, 169).

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Arthur M. NONOMURA: カリフォルニア バークレイ産緑藻 *Botryococcus braunii* var. *showa*（新種）について

Botryococucs braunii var. *shoaw* は，この属中最大の細胞で最大の集落を形成し得る。成長速度のはやいときは特徴ある小さなピーナツ殻状の集落を形成するが，成長速度のおそいときは，不定形の大きな細胞凝集体を形成する。有性生殖は認められない。この新変種は成長期に乾燥量の⅓に及ぶ botryococcene hydrocarbon を産生する。ジクロヘキサン環を一つもった炭素数32からなる botryococcenoid はこの変種のみにみられる。

(Scrips Institution of Oceanography, University of California).