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RESEARCH ARTICLE

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Fungus propagules in plastids: the mycosome hypothesis

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Abstract A stress-induced "mycosome" phase of *Aureobasidium pullulans* consisting of minute reproductive propagules that may revert directly to walled yeast cells is described. Mycosomes detected by light- and electron-microscopy reproduce within senescent plant plastids, and display three developmental pathways: wall-less cells (protoplasts), yeast cells, or membrane-bounded spherules that harbor plastids. Widespread in plant and algal cells, mycosomes are produced by both ascomycete and basidiomycete fungi.

Keywords *Aureobasidium pullulans* · Fungi · Plastids · Mycosomes · Someangia

Introduction

Minute fungus propagules that I call "mycosomes" are associated with algal and plant plastid development. I hypothesize that certain fungi have a cryptic life-history phase specialized to use the lipids and nitrogen of senescing plastids for their reproduction. Mycosomes are defined as wall-deficient reproductive propagules (diameter ca. 0.1–1.0 μm) that develop into larger forms: wall-less cells (protoplasts), yeast cells, or membrane-bounded thylakoid-containing spherules in which mycosomes reproduce. I discovered mycosomes when yeast cells cultured from the cytoplasm of diverse plants developed from minute precursors. Most of the enlarged unicells produced conidia and mycelia morphologically and physiologically similar to the pleiomorphic ascomycete *Aureobasidium pullulans* (ATCC strain 90393). Variously pigmented (black, olive green, yellow brown, rust brown or hyaline) on glucose-yeast nitrogen base agar [31], all isolates were fermentation-negative, urease-positive and utilized citrate [4] and ascorbate but usually not iso-ascorbate as a sole carbon source [3]. Well known as ubiquitous on aerial plant surfaces [2, 13], *A. pullulans* also has an endobiotic phase [23, 25, 26] that has not been described. Aureobasidin antibiotic production inhibits pathogenic fungi [14, 29]. A dothideaceous loculoascomycete, *A. pullulans* is either a species complex, or an unusually variable asexual state of *Discosphaerina fulvida* [32]. In this study I seek the developmental cycle of mycosomes, including their plastid relationship. If plant-derived mycosomes give rise to *A. pullulans* yeast, then axenic fungal cultures should regenerate mycosomes capable of reversion to *A. pullulans*. I describe *A. pullulans* mycosome life history in vitro, and also some in vivo stages. Mycosome distribution was sought in diverse photosynthetic organisms.

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Materials and methods

Organisms and media

Plants

Albuca sp., *Cycas revoluta*, *Ginkgo biloba*, *Ornithologalum caudatum*, *Polytrichum* sp, *Sequoia sempervirens* (UC Irvine Arboretum); *Ephedra* sp. (Mohave desert, Calif.); *Phoradendron californicum*, *Cuscuta subinclusa*, *Castilleja stenantha*, marine intertidal *Zostera* sp. (Orange County, Calif.); *Cassytha filiformus* (Ohau, Hawaii); *Pilostyles thurberi* (Texas); *Lycopodium* sp. (Wards Natural Science Biologicals, Rochester, N.Y.).

Algae and cyanobacteria

Culture Collection of Algae at the University of Texas (UTEX): *Anabaena flos-aquae* 1444, *Lyngbya kuetzingii* 1547, *Cylindrospermum licheniforme* 2014, *Trebouxia erici* 910, *Glaucocystis nostochinearum* 64, *Chlorella kessleri* 2229, *Chlamydomonas reinhardtii* 2337, *Nanochloris eucaryotum* 2502, *Prototheca kruegeri* 329, *Trentepohlia* sp. 1227, *Klebsormidium flaccidum* 321, *Zygnema circumcarinatum* 1559, *Mesotaenium caldariorum* 41, *Spyridia filamentosa* 1508, and *Phaeodactylum tricornatum* 640; UC Davis

research collection: *Coleochaete scutata* 262; Wards Natural Science Biologicals: *Mougeotia* sp. W 0280, *Zygnema circumcarinatum* W0900, *Dunaliella salina* W 0171, *Navicula pelliculosa* W 1210, *Tribonema aequale* W 1400; Connecticut Valley Teaching Collection: *Spirogyra* sp., *Chlamydomonas* sp.

Media

Media contained the following components in 100 ml sterile distilled water:

AC	0.1 g ammonium nitrate + C (0.02 g citric acid, pH ca. 4.5)
E	10^{-6} M ethylene, from 2-chloroethyl-phosphonic acid
ACE	AC + E
ACEG	ACE + G (2 ml glycerol)
ACPc	AC + 0.5% L-α-phosphatidylcholine
B	0.3 g bovine serum albumin (fraction V; Sigma, St. Louis, Mo.)
BG	B + G
BGE	BG + E
BMG	B + M (10 g mannitol) + G
BMGYM	BMG + 2 g Difco YM broth (Difco, Detroit, Mich.)
BAC	B + AC
BCP	B + C + 50 ml prune extract (five sliced prunes boiled in 500 ml water, extract filter-sterilized 0.2 μm)
MSk	10% Murishige-Skoog minerals + B + 0.3 g $CaCl_2$ + 0.6 g $MgSO_4$
MSk-2	10% MSk minerals + E + B + 0.1 ml glycerol
YMA	Difco yeast-maltose broth + 2% agar

Media were autoclaved or filter-sterilized as necessary. Gelman cellulose filters of 0.2 μm-pore diameter were used to attempt mycosome separation from plant and fungus cytoplasm. Media containing plant or fungus cytoplasm was filtered through tandem (5.0 μm above, 0.2 μm below), autoclaved 25 mm filter-holders attached to a 200 ml sterile plastic syringe.

Mycosome-phase from plant cytoplasm

Psilotum nudum, a leafless vascular plant, served as a primary experimental host. The mycosome phase develops in *Psilotum* plastid-rich cytoplasm (prc) expelled by glass rod maceration from surface sterilized, water-stressed, post-reproductive stem tissue, and transferred to water or other liquid media.

Experimental techniques

Tissue senescence

I conditioned plant tissue and algal cells in cold water, or acidic AC medium, sometimes containing ethylene (E or ACE), because exogenous ethylene, water stress and prolonged refrigeration promote a sudden rise in plant ethylene production and accompanying senescence symptoms, particularly plastid yellowing [30]. Tissue age and acidic hydration (ca. pH 4.5) are key mycosome-induction factors. Surface-infected young (dark green) *Psilotum* stems do not yield yeast cells, whereas most older (yellowish green, naturally dehissed sporangia) samples are yeast-positive after acidic hydration. Prc samples from both young and older stem segments usually yield *A. pullulans* prior to surface sterilization, and are negative for fungi when cultured in YM medium immediately after sterilization. When sterilized cohorts of the two age classes are conditioned for 48 h in AC medium before prc samples are added to YM medium, young stems continue to be negative for yeast, whereas most older stem samples yield *A. pullulans* unicells.

Verification of sterility

All work was performed under a laminar-flow hood, with sterile technique. Surface and internal forms of *A. pullulans* are distinguished as follows. Surface organisms are killed by stem submersion for 10 min in 20% Chlorox bleach followed by three washes in sterile distilled water (dH_2O). Post-reproductive *Psilotum* stems with dehissed sporangia are cut into 2 cm segments and mixed prior to selecting three for each prc sample. Surface sterilization efficacy is verified when prc samples cultured 48 h in liquid yeast-maltose medium (YM; Difco) are negative for fungi. The mycosome phase is induced by conditioning the remaining cohort of sterilized stem segments in dH_2O for 3–7 days in the refrigerator or 48 h in acidic AC medium at room temperature. After conditioning, stem segments are rewashed and macerated in a few drops of dH_2O, and the prc pipetted into a shallow layer of dH_2O (or other medium). Upon settling in Corning 50-mm plastic plates with tight-fitting lids, the chloroplasts form a thin covering over the container bottom. Culture samples monitored by light microscopy are yeast-negative for approximately 24 h. Between 36 and 48 h, a single sample may contain hundreds of small yeast cells, far in excess of numbers that could be generated by rare surface cells that may escape sterilization. Tissue age differences and mycosome mortality from excessive sterilization may produce inconsistent results.

Green plastids were cultured because they retain membrane integrity and respond differently than senescent forms. Prc from green post-reproductive stems refrigerated 7 days in water was cultured in BMGYM on YMA (Fig. 3k, l). To determine whether green plastids could be conditioned after cytoplasm release from stems, a single prc sample from a fresh post-reproductive green stem was subdivided into three treatments: (1) 96 h in BMGYM (non-conditioned control), (2) 96 h in acidic BCP, and (3) 48 h in BCP, followed by 48 h in BMGYM (Fig. 3d–f).

Mycosomes from A. pullulans pure cultures

Single-cell clones of *Psilotum*-derived *A. pullulans* were stressed in dH_2O, AC medium, ethylene, antibiotics, dye, or combinations of these, and macerated as per plant cells. To test mycosome longevity, *A. pullulans* cells were inoculated into flasks with dH_2O containing erythromycin, ampicillin, L-canavine or amphoterecin-B at 0.5 mg/ml. After 14 months at room temperature, water samples were observed (Fig. 1k, l) and transferred to ACEG on YMA.

Electron microscopy

Samples were fixed in 4% glutaraldehyde, post-fixed in 2% osmium tetroxide/0.1 M PO_4, and plastic embedded. Thin sections stained in 2% uranyl acetate followed by lead citrate were observed with a Zeiss EM 10 microscope. To stabilize mycosome and yeast cell dissociation from fragmenting senescent plastids during fixation for EM, I cultured prc in modified MSk medium ($-CaCl_2/MgSO_4$, + 0.1 ml glycerol) on glass disks, fixed the material at 24 h, and embedded the surface with agar to prevent movement (Fig. 5g).

Light microscopy

Color images are unstained or stained with aniline blue (AB) in lactophenol for fungi, I_2KI for starch, sudan black (SB) for lipid, or fluorescent 4′, 6-diamidine-2-phenylindole (DAPI)-DNA stain, and photographed in transmitted light, phase contrast or UV on a Zeiss Axiophot photomicroscope. Areas of concentrated AB stain appear red under phase contrast.

Mycosome-phase from algal cytoplasm

I sampled three cyanobacteria and 12 algal axenic cultures from UTEX, and a research culture of *Coleochaete scutata*. The UTEX vials were stressed for 6 weeks under Growlux lamps (16-h days);

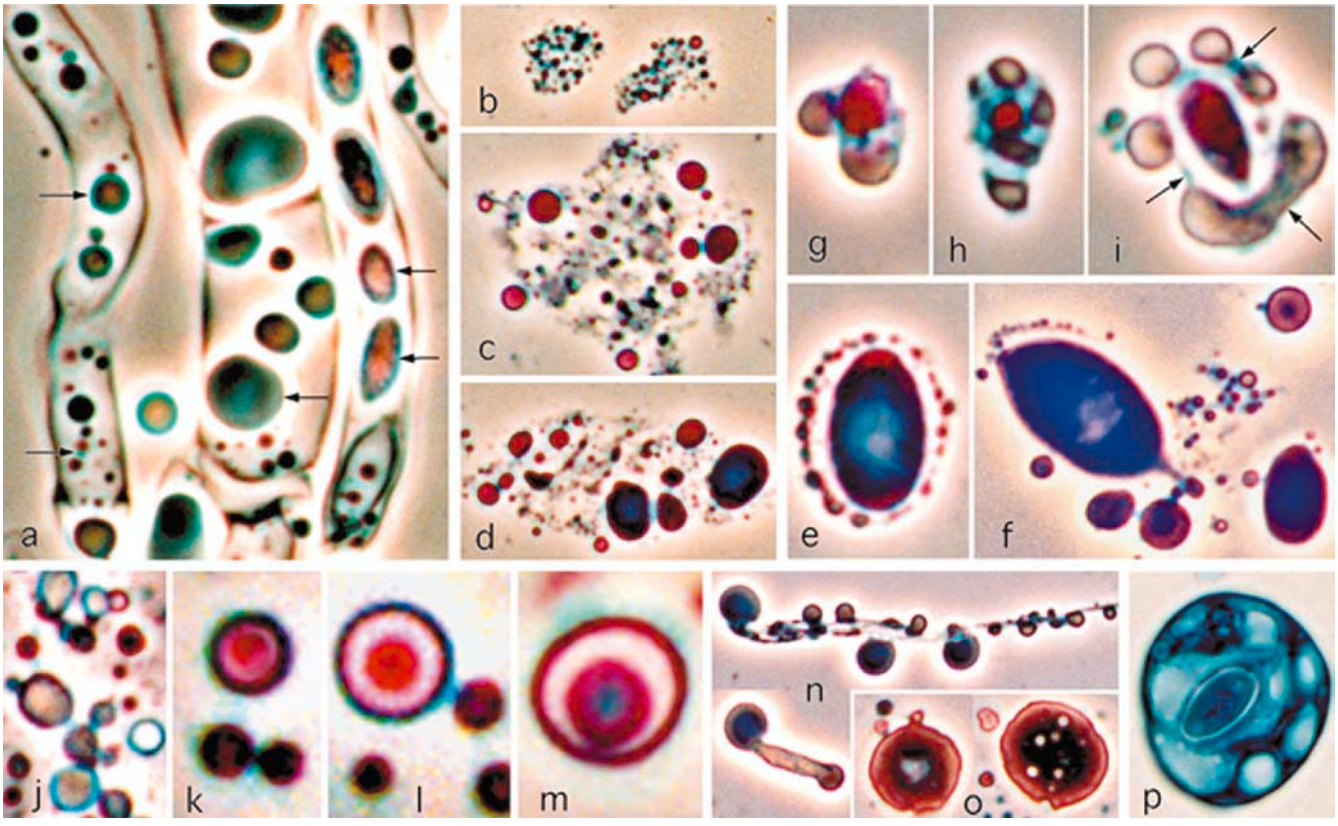

Fig. 1 Mycosome (ms) developmental stages from pure cultures of *Aureobasidium pullulans* (**a**–**l**) and *Psilotum nudum* (**m**–**p**). **a** Budding ms in hyphae (*left*), "lipid bodies" (*center*) and developing conidia (*right*) [×2,000, aniline blue (AB) + Sudan black (SB)]. **b**–**j** ms and yeast recovered from 0.2-μm filtration: **b** two someangia with numerous ms (×1,250); **c** densely staining ms (*red*) that **d** form yeast cells (×2,300). **e**–**i** Yeast development within someangia (×3,500): **e** a yeast within the someangium; **f** a fragmented someangium that produced multiple yeast. Note transition from spherical to ovoid shape; **g**, **h** ms produce nonstaining forms within a membrane; **i** the central ms develops as a yeast cell, and the someangium constricts (*arrows*) around the peripheral ms. **j** "Empty" and nonstaining ms (×2,200). **k**–**m** Ms bud and enlarge as spherules containing one or more ms (×8,000). **n** Spherules produce wall-deficient hyphae (×1,250), or **o** may accumulate lipids (×2,000, SB + AB). **p** Ms released by narrow constricting filaments enlarge as protoplasts that form conidia (×1,000). Experimental conditions: **a** *A. pullulans* (Ap) 5 days in AC (for definition of media, see Materials and methods), +10 days in AC over BMGYM agar; **b–f, j** Ap 14 days in AC, filtered 0.2-μm into ACE on YMA, transferred to fresh ACE on YMA at 5 days; **g**–**i** Ap 5 days in AC, medium cheesecloth-filtered into ACEG for 48 h; **m**–**o** *P. nudum* (Pn) plastid-rich cytoplasm (prc) in AC, filtered 0.2-μm onto YMA for 5 days (**m**, **n**) and 21 days (**o**); **p** Pn 48 h in dH_2O, prc 60 h in BMG on YMA

the cells were then conditioned for 48 h in 10^{-6} M ethylene, macerated with a sterile glass rod, plated in modified MSk medium on agar, and observed from 48 h to 16 days. A second sample of *T. erici* 910 obtained from UTEX 1 year later was macerated in ACPc medium and plated over YMA for nearly 6 months. Non-sterile algal cultures obtained from commercial teaching collections were also senesced, macerated and cultured. Fungal isolates were identified to genus using Yeast-IDent (API, Plainview, N.Y.).

Results

Mycosome-phase from *A. pullulans*

Conditioned in sterile distilled water, AC medium or antibiotics, *A. pullulans* produces internal conidia indistinguishable from external conidia, and minute AB-staining bodies hypothesized to be mycosomes. Mycosome-cycle stages also become prominent when AC-treated cells are transferred to fresh AC over rich (BMGYM) agar. These hyphae develop mycosomes and budding spheres (Fig. 1a, left arrows), apparent lipid bodies (center arrow), and developing conidia (right arrows). The mycosome latency test showed that yeast cells could be recovered from all four water + antibiotic cultures after 14 months at room temperature, and samples examined prior to plating revealed large numbers of mycosomes (Fig. 1k, l).

Mycosomes derived from fungus pure cultures rarely transform to yeast cells in conditioning media. Reversion of mycosomes to yeast may occur within 24 h after transfer of a few drops of these media to YM agar covered with fresh AC medium containing ethylene, phosphatidylcholine or prune extract. Mycosomes from *A. pullulans* (Fig. 1b–i) and plant prc (Fig. 1m–p) pass through 0.2 μm filters, but not in large numbers. Since detection relied on subsequent transformation to yeast (medium-dependent), frequency was not quantified. *A. pullulans* mycosomes filtered (0.2 μm) into ACE on YM agar enlarged to become structures I call

"someangia" (Fig. 1b–d). Someangia are defined as membrane-bounded acytoplasmic protoplasts that produce mycosomes within invaginating membranes. The mycosomes may stain densely (red) with AB (Fig. 1b, c). They become oval and less dense (blue) as they mature (Fig. 1d). A single mycosome may divide within an expanding protoplast membrane (Fig. 1g, h) and later enlarge as a yeast cell (Fig. 1i). The peripheral non-staining mycosomes may be released by constriction of the protoplast membrane (Fig. 1i, arrows). The peripheral mycosomes remain undeveloped (Fig. 1e), or enlarge as yeast cells that cause fragmentation of the someagenic protoplast (Fig. 1f).

Mycosomes also enlarge as distinctive membrane-bounded spherules that appear empty or contain material that does not stain with AB (Fig. 1j). Spherules usually contain a single mycosome (Fig. 1k)—in rare cases two to three—within a central area that becomes non-staining as the budding spherule enlarges (Fig. 1l). The parent spherule displays a densely staining ring-like peripheral cytoplasm (Fig. 1m) that narrows with age. I interpret spherules and someagenic protoplasts to be alternative forms of a common mycosome progenitor. Spherules may produce a single yeast cell (not shown), wall-deficient hyphae (Fig. 1n), or lipid-accumulating forms that bud mycosomes (Fig. 1o). Reticulate narrow filaments partition into linear arrays of independent mycosomes that may enlarge as vacuolate protoplasts that produce internal conidia (Fig. 1p).

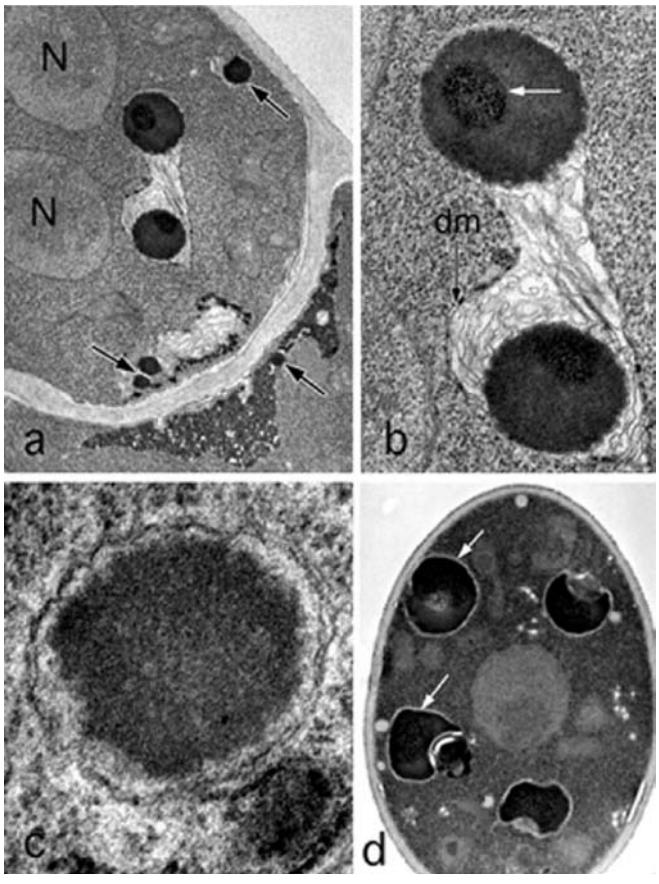

Fig. 2a–d Mycosomes within *A. pullulans* cells. **a** Ms (*arrows*) enlarge as electron-dense bodies within double membrane-bounded organelles called someangia. Note ms are smaller than nuclei (*N*), (×8,000). **b** Double membrane (*dm*) with membrane lamellae and two ms, each with a prominent inclusion (*arrow*) (×21,000). **c** Mycosomes develop cell walls internal to the double membranes (×18,000). **d** Someangia accumulate lipid material and appear as osmiophilic bodies surrounded by an electron-lucent "line" (*arrows*), the space between the membranes (×8,200). Experimental conditions: Ap cells from Pn prc 48 h in BMGYM on YMA (**a**, **b**, **d**) and *Cuscuta* cytoplasm 40 h in dH_2O on YMA (**c**)

Mycosome ontogeny

A. pullulans cells from prc produce mycosomes within membrane-rich, double membrane-bounded organelles (Fig. 2a, b). I interpret these as homologs of the someangia I observed in culture (Fig. 1b). Small electron-opaque bodies (Fig. 2a, arrows) appear to originate within the double membrane envelope and to move into the membrane lamellae. They enlarge as spherical mycosomes; some show a heterogeneous electron-opaque inclusion (Fig. 2b, arrow). Single mycosomes bud from the someangium and develop a wall internal to its two bounding membranes (Fig. 2c). The someangia of yeast cultured in YM medium contain lipid and degenerating mycosomes, or appear as osmiophilic bodies (Fig. 2d).

Mycosomes within plastids and plastids within fungus protoplasts

Plastids cultured from prc contain mycosomes. Plastids also occur within fungus protoplasts. Each experimental outcome is sensitive to plant tissue age, conditioning treatment and the medium in which the cytoplasm is placed. Well-senesced plastids observed immediately after stem maceration produce abundant plastoglobuli (Fig. 3a, double arrows). I define two types of plastoglobuli: lipid bodies that develop in the plastid stroma and contain mostly neutral lipids, and gray spherical bodies with a high glycolipid content that are enclosed by a membrane structurally similar to the outer monolayer of thylakoid membranes [22]. I postulate that the latter contain mycosomes that enlarge as spherules (Fig. 3a, arrow) similar to those from *A. pullulans* (Fig. 1n). Larger forms contain multiple mycosomes (Fig. 3b, arrows) and some of these produce yeast (Fig. 3c).

Although healthy green plastids are typically resistant to mycosome development, green plastids conditioned 48 h in BCP medium, followed by the addition of 50% rich medium for 48 h showed mycosome to yeast transformation ($n = 4$, Fig. 3f). Controls exposed only to acidic BCP (conditioning medium) for 96 h were negative for yeast, but the mature plastids contained many small dense bodies as well as larger spheroidal bodies I interpret to be growth-arrested yeast cells (Fig. 3d, e; arrows). Non-conditioned controls cultured 96 h in rich medium showed no signs of mycosome development, as

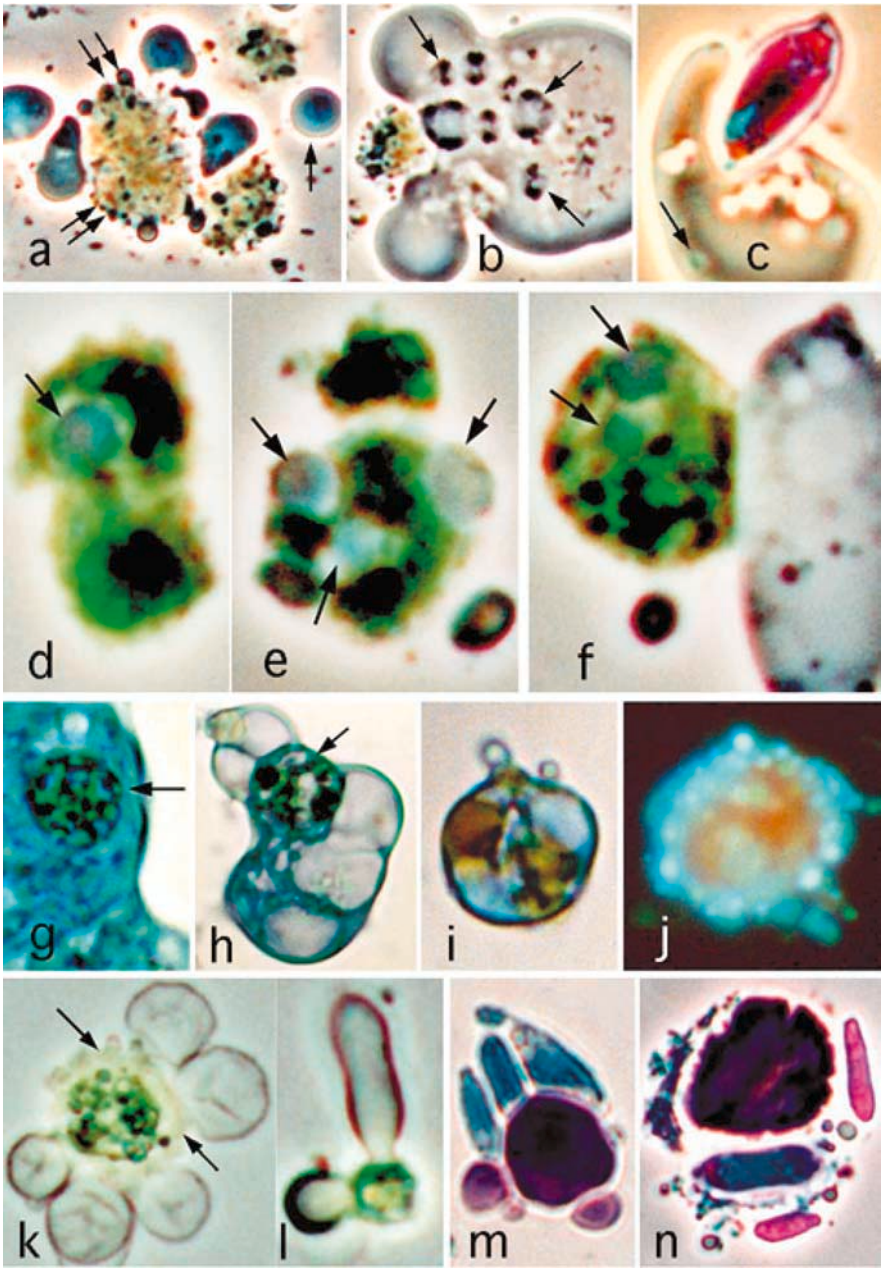

Fig. 3a–n Mycosomes develop within plastids that sometimes occur within a fungus membrane or protoplast. **a** Ms enlarge from the plastoglobuli (*double arrows*) of senescent plastids (×1,000). **b** Lipid bodies contain ms (×1,000). **c** A yeast cell from a lipid body that contains ms (*arrow*) (×2,000). **d–f** Mycosome-to-yeast transition in green plastids (×3,000). **g** A *Psilotum* plastid (*arrow*) within a fungus protoplast (×1,000). **h** A fungus protoplast extends from the plastid margin (×800). **i** A plastid enclosed by an AB-staining, budding membrane (×1,200); **j** A 4′, 6-diamidine-2-phenylindole (DAPI)-stained plastid enclosed by blue-fluorescing fungus membranes containing ms-sized bodies with DNA-like fluorescence (×1,500). **k**, **l** Spherical or elongate protoplasts develop from the margin of green plastids (×1,800). **m**, **n** Yeast develop at the margin of plastid starch (×1,250). Experimental conditions: **a, b** Pn 24 h dry, 77 h in B, prc observed at maceration; **c** Pn prc 5 days in BGE; **d–f** see Methods; **g–j** as in Fig. 1p; **k** and **l** Pn 6 days cold in dH_2O, prc 48 h in BMGYM; **m** Pn 48 h in AC, prc 5 days in BMGYM; **n** Pn 7 days cold in dH_2O, prc 40 h in ACEG

expected. Rich medium is universally inhibitory prior to acidic conditioning. BCP was apparently a key stimulant for mycosome development within green plastids. In the absence of senescence and in vivo conditioning, no chloroplasts occurred within a fungus membrane or protoplast. Individual plastids may have been infected with mycosomes during in vitro conditioning. Filter-sterilized (0.2 μm) prune extract cannot be excluded as a mycosome source.

Chloroplasts also developed within AB-staining protoplasts cultured in BMG medium at pH >6.5. For reasons unknown, these robust conidia-producing forms (Fig. 1p) were not observed after 1986. The protoplasts included 80×130 μm diameter units with senescent lipid-rich plastids and 15–20 conidia, others with green plastids (Fig. 3g), single plastids with protoplast extensions (Fig. 3h), or single plastids enveloped by a blue-staining membrane with small "buds" (Fig. 3i). Under UV light, plastid thylakoid membranes autofluoresce red or red-orange, and DAPI-stained fungus cells and protoplasts fluoresce blue and show bright spots that are presumably nuclei. Bright DAPI fluorescence was seen within blue membranes that enclose red-orange plastids (Fig. 3j). These figures graphically support my hypothesis that individual plastids develop within a mycosome-containing fungus membrane or protoplast (see also Fig. 5). Conditioned in vivo and cultured in rich medium, green plastids produce multiple globose or elongate protrusions (Fig. 3k, l) that may indicate aberrant mycosome development stages. In Fig. 3k, note that the

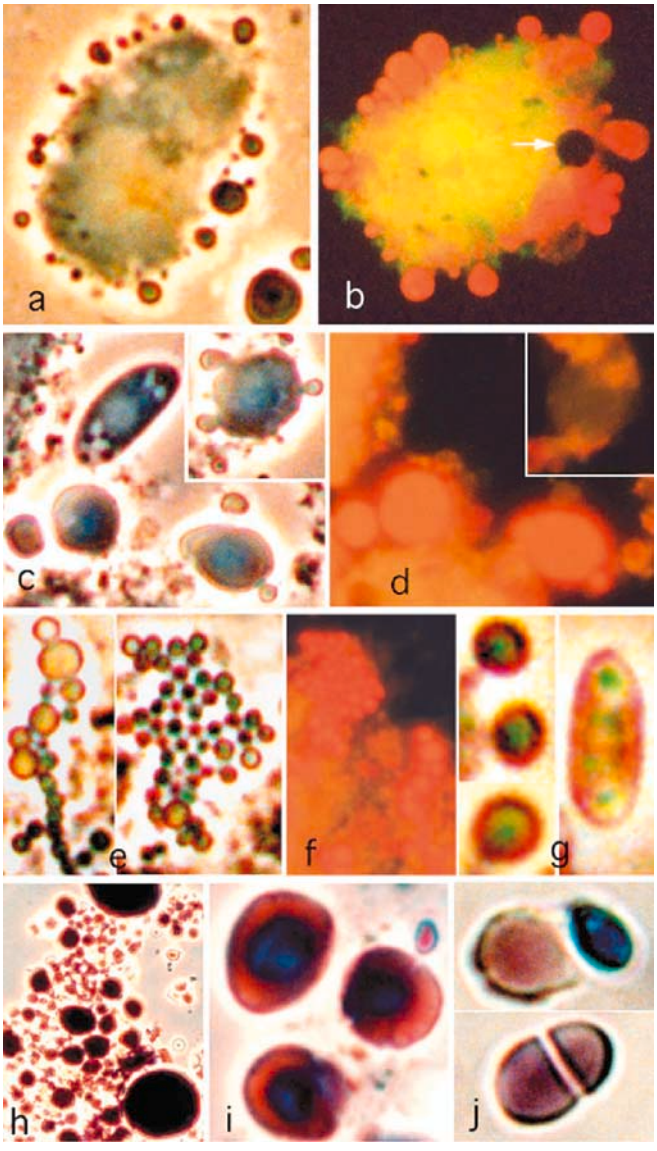

Fig. 4a–j Mycosomes contain plastid pigments and starch. **a** A fungus protoplast that contains plastids, with ms at its margin (×2,000, no stain). **b** A similar fungus protoplast whose plastids fluoresce yellow under UV illumination. External ms fluoresce orange. Starch grain at *arrow* (×1,200); ms stages seen with **c** phase contrast and **d** UV microscopy. Budding yeast precursors show variable fluorescence, whereas yeast do not fluoresce (×2,500). **e** Mycosomes are green in the center (also **g**), become yellow with increasing size (×3,800), and (**f**) autofluoresce (×2,000). **g** Some yeast contain green inclusions assumed to be plastids (×8,000). **h** Mycosomes containing starch, from macerated *A. pullulans* cultured in prc (×300). **i** Some starch-producing ms contain an AB-staining body (×2,500). **j** Starch-producing ms in the process of division, one produces a yeast cell (×1,400). Experimental conditions: **a**–**g** Pn 8 days cold in dH_2O, prc 48 h in dH_2O; **h** Pn prc filtered 1.0-μm in B, plus Ap treated 9 days in B, 24 h development; **i** as in Fig. 3n; **j** Pn 48 h in MSk-2, prc 48 h in MSk-2

membrane associated with the protrusions (arrows) is separate from the chloroplast. Mycosomes were also detected within membranes that envelope iodine-stained starch [33]. Yeast cells develop primarily at starch grain margins, either within barely visible membranes (Fig. 3m), or within narrow protoplasts that surround a central starch grain (Fig. 3n).

Mycosomes from plastids have plastid traits

Fungus protoplasts that contain senescent plastids (Fig. 4a) autofluoresce yellow under UV illumination (Fig. 4b). The peripheral mycosomes fluoresce orange, an indication of the presence of chlorophyll and/or carotenoids, presumably of plastid origin. Fungus membranes fail to autofluoresce; they appear greenish against the yellow-fluorescing plastids. Compared in phase contrast and UV (Fig. 4c, d), the orange-fluorescing mycosomes may produce fluorescing buds, show weak fluorescence and nonfluorescing buds (inset), or produce nonfluorescing yeast. Mycosomes from fully senesced plastids show green centers when small, they become yellow with increased size (Fig. 4e), and fluoresce orange under UV (Fig. 4f).

Viewed with phase contrast microscopy, the larger mycosomes may contain a single green body. Such green bodies are occasionally seen in yeast cells (Fig. 4g), suggesting that mycosomes that become yeast contain plastids that remain briefly photosynthetic within their walls. The plastid-within-mycosome hypothesis is consistent with the fact that mycosomes often produce reddish-staining starch (Fig. 4h) similar to the amylopectin component [24] of plant starch. Although some fungi do produce a purple-staining amylose-starch, fungus starch lacks amylopectin. Sectioned for EM, the starch-producing mycosomes show a wall-like boundary and an electron-dense center without defining characteristics [33]. Some double-stained mycosomes show a well-developed AB-staining body enclosed by iodine-staining starch (Fig. 4i). Similar mycosomes were recovered from *A. pullulans* pure cultures on two occasions. Yeast cells also develop as division products of starch-producing mycosomes (Fig. 4j, top), illustrating the duel developmental potential of this fungus propagule.

Plastids enclosed inside fungal protoplasts

Plastids cultured from *Psilotum* were often enclosed by a distinctive electron-opaque boundary considered to be a fungus protoplast fused with the outer plastid membrane (Fig. 5a). These contain electron-dense and larger opaque bodies that are indistinguishable from plastoglobuli at low magnification (Fig. 5a). Enlargement may show a membrane-like boundary, heterogeneous content, division-like images and clustered grouping (Fig. 5b, d). Mycosomes are typically associated with thylakoid membranes (Fig. 5c), and many show spherical structures (arrow) that are common elements within derived forms (Fig. 6a, b; arrows). Intermediate stages (Fig. 5e) were infrequent, yet several small walled cells, between 0.5–1.0 μm in diameter (Fig. 5f), were found associated with fragmenting thylakoid membranes and osmiophilic

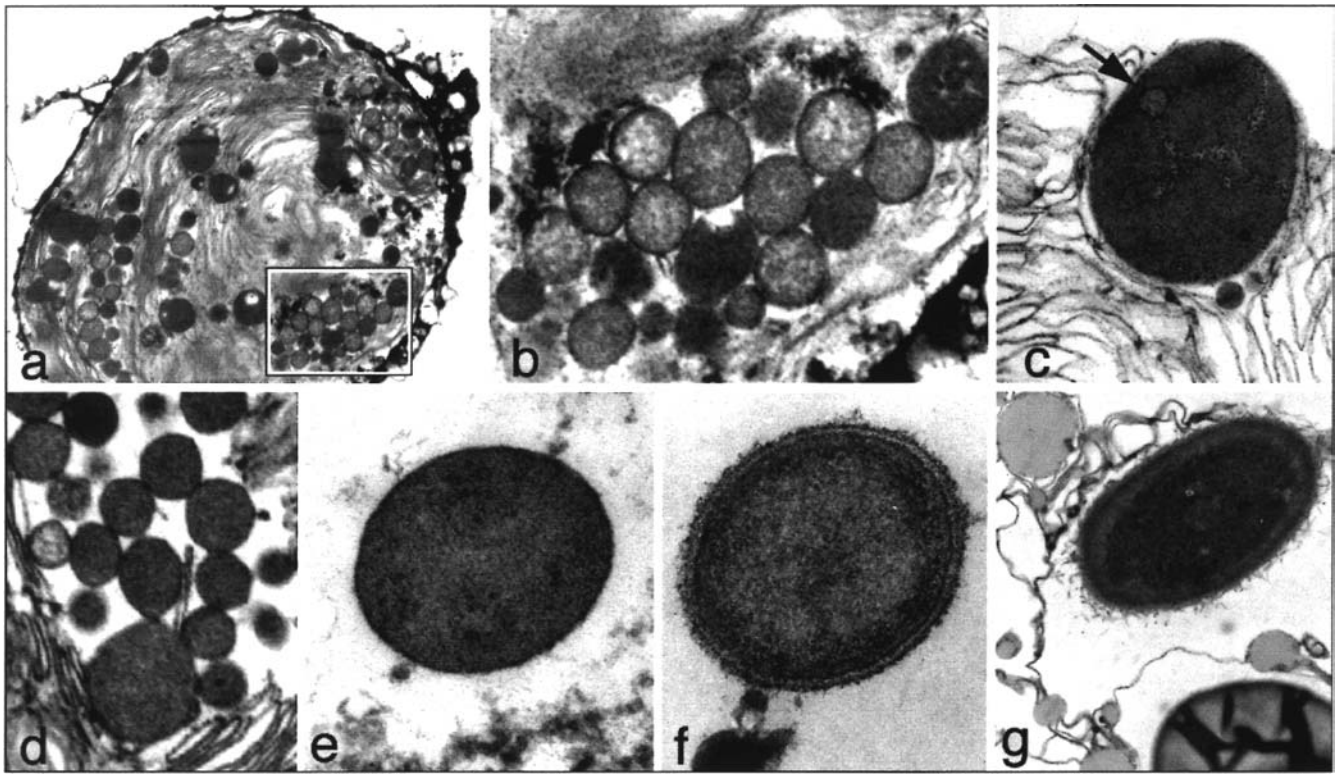

Fig. 5a–g Mycosomes and yeast from *Psilotum* plastids, transmission electron microscopy. **a** An electron-opaque protoplast surrounds a plastid that contains numerous ms (×13,000). **b** Enlargement of inset ms cluster from **a** (×45,500); **c** Mycosomes associated with plastid thylakoid membranes often show spherical structures (*arrow*, see also Fig. 6a, b) (×34,600). **d** Typical ms size range (0.2–0.5 μm) during their division (×32,000). **d–f** Postulated spherical to ovoid developmental sequence from ca. 0.2 μm (**d**) to 0.40 μm (**e**), to a walled yeast 0.8 μm in length (**f**), (**e**, ×80,000) (**f**, ×47,000). **g** An *A. pullulans* yeast attached to the thylakoid membranes of a senescent amyloplast immobilized during fixation (×11,100). Experimental conditions: **a–f** Pn 24 h dry, 24 h in dH_2O, prc 45 h in BMGYM; **g** see Methods

bodies. In an experiment designed specifically to prevent movement during fixation, agar embedding of prc cultured on glass disks produced the profile in Fig. 5g, one of several yeast cells found attached to the thylakoid membranes of degenerating plastids with starch.

Cultured plastids show predominantly electron-dense mycosomes (Fig. 6a, b) containing heterogeneous spheroid areas defined by lighter density, plus spherical structures (arrows). These mycosomes may divide to produce ring-like forms (Fig. 6b) I identify as plastids that contain mycosomes (pcm): pcm also enlarge from electron-opaque bodies < 0.2 μm in diameter, situated within the parent plastid envelope or, more frequently, associated with thylakoid membranes; pcm show central fragments and strands that emanate from a narrow peripheral cytoplasm (Fig. 6b, c); pcm in the envelope may be enclosed by a fungus membrane fused with the outer plastid membrane (Fig. 6 c, arrow); pcm are also produced within protoplast extensions (Fig. 6d, asterisks) that are continuous with the outer plastid membrane. The pcm "envelope" contains numerous electron opaque units, one or more of which enlarge as plastoglobuli (Fig. 6d, arrows) that are indistinguishable from those within the parent plastid. Independent pcm released from *Psilotum* plastids contain thylakoid membranes and mycosomes (Fig. 6e, f), or may be packed exclusively with mycosomes that appear to divide inward from the envelope. The large mycosome within the pcm seen in Fig. 6f is indistinguishable from yeast that develop abnormally (Fig. 6g); pcm genesis from minute electron opaque bodies may also occur in small protoplasts that originate from plastid thylakoid membranes (Fig. 6h). The plasma membrane-associated bodies (Figs. 6h, i, arrows) enlarge as pcm, identified by their electron-opaque membrane-bounded bodies (Fig. 6i, double arrow). The pcm bodies (Fig. 6j, arrows) apparently divide inward and enlarge as plastoglobuli associated with thylakoid membranes (Fig. 6j, right). Small pcm about 1.0 μm in diameter may produce single starch grains as well as thylakoid membranes.

Mycosome phase from algal monocultures

Trebouxia erici, *Coleochaete scutata* and *Phaeodactylum tricornatum* extracts yielded basidiomycete yeast (Table 1). *Cryptococcus* and *Rhodotorula* species are known to live inside plants [16, 25]. The transition from mycosome to yeast was documented (light micrographs) in cultures of both *T. erici* and *C. scutata*. Although the remaining UTEX cultures were yeast-negative, it is significant that typical mycosome stages were present in most cases.

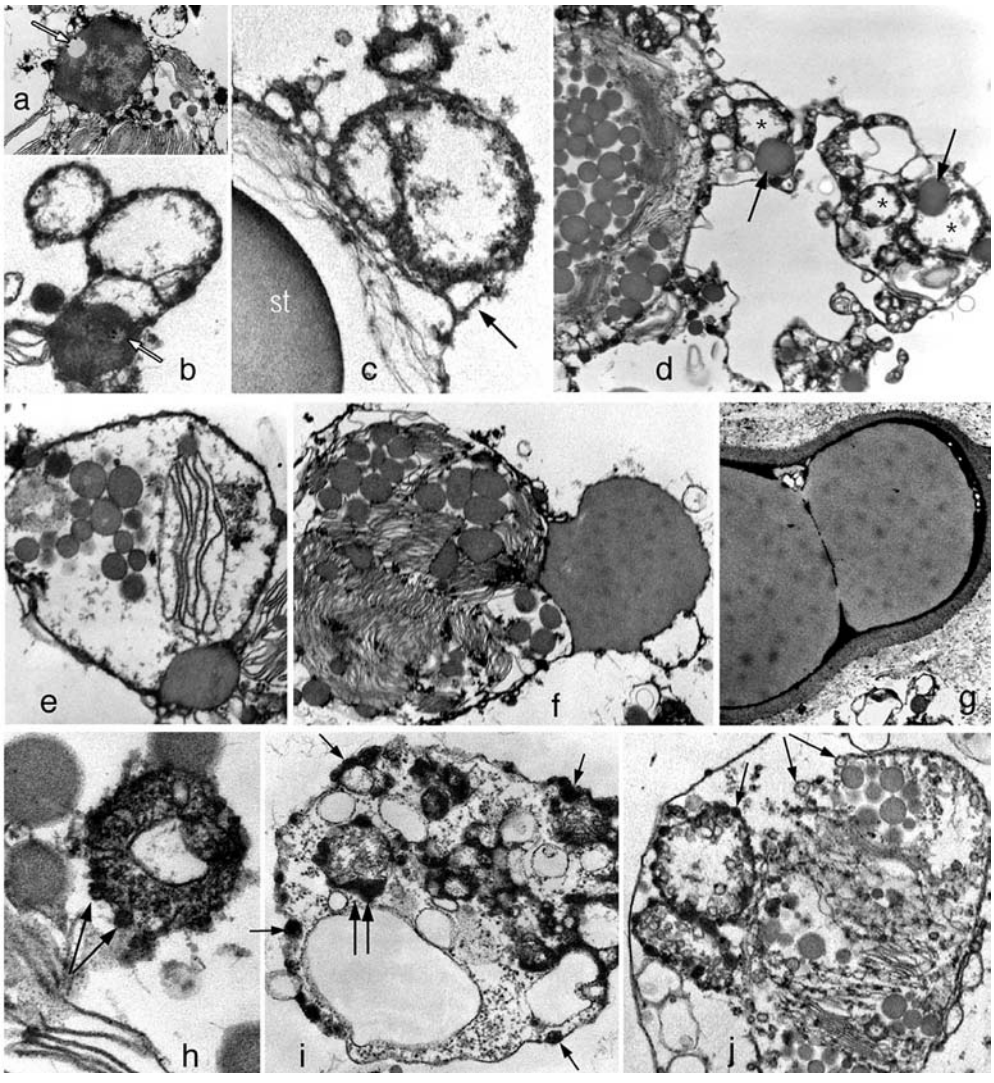

Fig. 6 *Psilotum* plastids produce plastids containing ms (pcm) (**a**–**f**) and fungus protoplasts that also produce pcm (**h**–**j**). **a** A ms with a spherical structure (*arrow*, also **b**) and lighter gray areas, attached to thylakoids (×12,000). **b** A dividing pcm that came from a thylakoid-associated mycosome (×21,000). **c** A pcm within a plastid envelope (*st* starch, ×32,000). **d** pcm (*asterisks*) with plastoglobuli (*arrows*), within a protoplast extension continuous with the outer plastid membrane (×14,000). **e** A pcm with thylakoid membranes and a membrane-bounded ms (×26,000). **f** A pcm (×11,000) contains an asymmetric ms indistinguishable from a yeast cell (**g**, ×12,000) that developed abnormally. **h** A fungal protoplast with plasma membrane-associated bodies (*arrows*), from a *Psilotum* plastid (×56,000). **i** A fungal protoplast with bodies (*arrows*) that enlarge as pcm (*double arrows*, ×24,000); **j** Maturing pcm with membrane-bounded ms (*arrows*) that divide into the stroma and enlarge as plastoglobuli (×21,000). Experimental conditions: Pn 72 h in dH_2O, prc 46 h in BMGYM

Cyanobacteria (*Anabaena flos-aquae* and *Cylindrospermum licheniforme*) produced mycosomes and spherules at the margins of senesced cells after 5 months, similar in all respects to forms derived from plants. Mycosomes that stained with AB were abundant in the periplasm of senescent *Glaucocystis* cells and some plastids (cyanelles) budded similar bodies from narrowed extensions. *Prototheca*, a colorless alga, yielded typical mycosome-containing membranes, and large AB-staining spherules clustered along thin filaments. *Trentepohlia* plastids accumulate a bright yellow β-carotene that provides a distinctive plastid pigment marker. Extract from this lichen alga produced reticulate membranes with mycosomes enlarging as bright yellow, dark-centered spherules, consistent with plant observations that mycosomes contain pigmented lipids similar to the plastids of their host organism.

Extract from *T. erici* 910 in ACPc medium produced an unusually compact, slow-growing mound of black filaments apparent only after 22 weeks of culture. *T. erici* 910 is a lichen alga, isolated from *Cladonia cristatella* by V. Ahmadjian (Clark University, Worcester, Mass.) and contributed to UTEX in 1975. Ahmadjian identified my fungus isolate as a variant of his original lichen fungus from *C. cristatella*.

In contrast to UTEX axenic cultures, yeast were readily isolated from several non-sterile algal cultures. Because algae cannot be surface sterilized, only yeast that associated with mycosomes are listed in Table 1. Mycosomes were observed within senescent algal cells in cases marked with an a.

Discussion

Plant-derived mycosomes transform to *A. pullulans* yeast capable of regenerating mycosomes that revert back to the typical walled phase. More complex than miniature yeast cells, mycosomes are membrane-bounded fungal propagules that develop as walled cells, mycosome-producing protoplasts (someangia), or spherules with variable content. In *A. pullulans* pure cultures, the spherule may remain empty or develop one or more mycosomes. In contrast, spherules from senescent

Table 1 Photosynthetic organisms that yielded fungi associated with one or more phases of mycosome life history. *Ap Aureobasidium pullulans*, *Ca Candida* sp., *Cr Cryptococcus* sp., –ID unidentified but not Ap, *Lf* lichen-fungus, *Rh Rhodotorula* sp., *Tr Trichosporon* sp.

Plants	Fungus	Algae	Fungus
Cuscuta subinclusa	Ap	*Coleochaete* UCD 262 [a]	Rh
Castilleja stenantha	Ap	*Trebouxia* UTEX 910 [a]	Cr
Cassytha filiformus	–ID	*Trebouxia* UTEX 910	Lf
Phoradendron californicum	Ap	*Phaeodactylum* UTEX 640	Tr
Pilostyles thurberi	–ID	*Mougeotia* sp. [a]	Ca
Ornithologalum caudatum	Ap	*Spirogyra* sp. [a]	–ID
Albuca sp.	Ap	*Zygnema circumcarinatum* [a]	Tr
Zostera sp.	Ap	*Dunaliella salina*	Rh
Sequoia sempervirens	Ap	*Chlamydomonas* sp.	Rh
Ephedra sp.	Ap	*Navicula pelliculosa*	Ca
Ginkgo biloba	AP	*Tribonema aequale*	Rh
Cycas revoluta	Ap		
Polytrichum sp.	Ap		
Psilotum nudum	Ap		
Psilotum nudum	Rh		
Lycopodium sp.	Ap		

[a]Mycosomes were observed within algal cells

plastids and plastid-containing fungus protoplasts may contain a central green body, plastid pigments, or starch with high amylopectin content.

Mycosomes develop inside plant plastids that have been incorporated into a wall-less stage of the fungus. Proplastids may be enclosed inside fungus protoplasts, but it is not clear how these organelles acquire mycosomes. Mature chloroplasts that contain mycosomes produce yeast cells, protoplasts and pcm; pcm develop from small electron-dense bodies and may be homologous to spherules observed with light microscopy. The presence of plastids within mycosomes can be confirmed with molecular probes, as can the affinity of the DNA-containing mycosomes.

Mycosomes that develop into internal conidia are comparable to those that form without wall layers in continuity between the conidium and the parent cell (e.g., endogenous conidia). While endogenous conidia are unknown in ascomycetes, since meiotic ascospores that develop within the double membrane ascus vesicle [15] are typically endogenous [10], the genetic potential for the double membrane someangium is likely to be present in mycosome-forming fungi. Mycosome discovery has likely been obstructed by fungus culture under nutrient-rich (laboratory) conditions. Internal conidia are rarely reported for *A. pullulans* [11, 18], but "lipid bodies" are commonly seen. The *A. pullulans* someangium becomes densely osmiophilic on typical nutrient agar (Fig. 2d), masking the mycosomes within. Similar electron-opaque bodies noted in several dimorphic fungal pathogens [1, 6, 7, 8, 9] may be someangia.

Mycosome-producing fungi are probably widespread. The causative agent of Dutch elm disease, *Ophiostoma ulmi*, produces "microendospores" [19] that pass through 0.45- and 0.22-μm Millipore filters. Injected into trees, these clear filtrates produced typical disease symptoms, even several months later [20]. *Candida* species consistently produce viable units filterable through Seitz sterilizing pads, sintered glass filters, or Gelman cellulose filters of 0.2-μm pore diameter [17]. The microhyphae (minimal diameter 0.2 μm) of *Fusarium oxysporum* f. sp. *dianthi* [21] may be mycosome stages. Mycosome ontogeny to mature ascomycete and basidiomycete yeast should be induced in vivo, and described in detail by comparative transmission electron microscopy studies. In situ hybridization with rRNA targeted probes [13, 27], new imaging techniques [12] and DNA probes should identify mycosomes and their filamentous phase within plant cells and tissue. Fungus-specific nuclear markers [5, 28] are predicted to demonstrate nuclei in mycosomes and to track mycosome behavior in plastids with multiple nucleoids.

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