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Changes in chloroplast structure in lichenized algae

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Abstract
Chloroplast morphology represents a basic characteristic in the systematic classification of trebouxioid algae. However, in different ontogenetic, physiological and ecological stages chloroplasts may vary markedly. Various developmental states of two algal species (*Asterochloris* sp. and *Trebouxia incrustata*) isolated from four lichens (*Cladonia foliacea*, *Lecidea fuscoatra*, *Lepraria* sp., *Xanthoparmelia conspersa*) were examined by confocal microscopy for variations in chloroplast structure. Distinct differences were detected between the chloroplast structure of cultured and lichenized photobionts. Remarkable chloroplast changes were observed during the process of algal liberation from the lichen thallus.

Keywords: Confocal laser scanning microscopy, green algae, isolation, Lecanorales, mycobiont, pyrenoid, symbiosis, thallus fragment method

1. Introduction

Lichens are symbiotic organisms composed of a fungal partner, the mycobiont, and one or more photosynthetic partners, the photobiont, that may be either a green alga or cyanobacterium (Nash III, 1993). In symbiotic organisms, the partners are typically adapted to coexistence to varying degrees. The success of algae and fungi as lichen symbionts implies a set of specialized and possibly irreversible characteristics (Ahmadjian, 1992). Both symbionts undergo a variety of structural, physiological, and biochemical modifications as a result of lichenization (Galun, 1988). Cyanobacterial as well as green algal photobionts are changed by the influence of their fungal partner. Due to these changes, identification of photobionts directly in the lichen thallus is often impossible (Friedl and Büdel, 1996). However, the changes of the algal cells are not permanent and when the cells are freed from the fungal hyphae they revert to their original size and characteristics after several divisions (Ahmadjian, 1992).

Morphological differences between symbiotic and cultured photobionts have been reported in many studies (see references in Bubrick, 1988; further e.g. Ahmadjian, 1992; Davis and Rands, 1993; Tschermak-Woess, 1995a,b).

Commonly, a remarkable reduction of cell size is found in a majority of eukaryotic bionts. Filamentous algae and cyanobacteria are generally reduced to unicellular forms. Both sexual and asexual reproductive strategies of algae and the cell division of cyanobacteria are usually modified. At the subcellular level, modifications of size, structure, as well as the number and distribution of some organelles and cellular structures were detected (e.g. chloroplast, thylakoid, pyrenoid, pyrenoglobuli, dictyosomes, etc.). Changes in cell wall chemistry and structure, and in the production of a gelatinous sheath have been observed in several cases. Cell envelopes and internal structures may be also directly affected by penetration of haustoria.

The degree of structural modification is dependent upon the type of photobiont and on the specific relationship between two particular partners (closeness of mycobiont-photobiont contact). However, these modifications may also be evoked by environmental conditions and physiological status of the biont.

This paper presents a microscopical study of photobionts from four different lichen taxa. The aims were twofold: 1) to demonstrate differences in chloroplast structure between the lichenized and cultured form of symbiotic algae from the genera *Asterochloris* and *Trebouxia* and 2) to document changes in chloroplasts during the unprompted liberation of algae from lichen thalli.

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Table 1. Morphological and biological characteristics of investigated photobiont strains.

Characteristics	*Asterochloris* sp.		*Trebouxia incrustata*	
	Cultured	Lichenized	Cultured	Lichenized
Vegetative cells	Spherical (12.5–23 μm)	Spherical or ellipsoidal (5–17.5 μm, 10–11 × 15–17.5 μm; in *Lepraria* only spherical!)	Spherical (12.5–22.5 μm)	Ellipsoidal or ovoid (12.5–15 × 17.5–20 μm) in *L. fuscoatra*, predominantly spherical in *X. conspersa* (6.5–17.5 μm)
Cell wall	To 1 μm thick, wall thickening to 2.5 μm	–	To 1 μm thick, wall thickening to 2.5 μm	–
Chloroplast	Lobate: lobes simple at first, less or more incised and extended in terminal part; later thinner, curved and branched; prior to cell division (aplano/zoosporogenesis) in parietal position	Compact, crenulate (short picked lobes) or with shallow incised flat lobes	Lobate: crenulate (small rounded lobes) or with large oblong ridged lobes (incised to 1/4 of chloroplast average)	Compact to lobate (simple, less or more oblong lobes)
Pyrenoid	Mainly single, naked, occurring in most compact part of chloroplast, surrounded with more or less distinct starch area	Distinctly penetrated by one or more thylakoids	Single or several, naked; in young cells and in a part of mature cells surrounded with distinct starch area	Starch area often very large
Nucleus	Single, in parietal position (occurring in invagination of chloroplast)	–	Single, in parietal position (occurring in invagination of chloroplast)	–
Reproduction	Aplanospores: 64 (128) in sporangium, zoospores (narrowly to broadly drop shaped, 5–10 × 2.5–4.5 μm, with two anterior flagella to 12 μm in length, apical stigma)	Aplanospores: 4–16 in sp.	Autospores: 4–16 in sp., often with non-synchronous division; aplanospores: 32 (64) in sp.; zoospores (narrowly drop shaped, 4–5 × 3 μm, with two anterior flagella about 7 μm in length, small apical stigma)	Autospores: 4–8 in sp., non-synchronous division very frequent

Table 2. List of taxa used in this study, with collection information and GenBank accession numbers.

Algal species (fungal species)	Collection number	Algal GenBank accession number ITS	Actin
Asterochloris sp. (*Cladonia foliacea*)	CLAD 1	AM906016	AM906049
Asterochloris sp. (*Lepraria* sp.)	LEP 36	AM900493	AM906046
Trebouxia incrustata (*Lecidea fuscoatra*)	LEC 1	AM920666	–
Trebouxia incrustata (*Xanthoparmelia conspersa*)	PAR 1	AM920667	–

2. Materials and Methods

Material

The algal symbionts used in this study were isolated from four lichens with different types of thalli and systematic position: *Cladonia foliacea* (thallus heteromerous dimorphic; Cladoniaceae), *Lecidea fuscoatra* (heteromerous crustose; Lecideaceae), *Lepraria* sp. (homoiomerous leprose; Stereocaulaceae; in the collected specimen two species were detected: *Lepraria nylanderiana* and admixed *Lepraria* sp.), *Xanthoparmelia conspersa* (heteromerous foliose; Parmeliaceae).

All lichens were collected from the single rocky steppe slope near Máslovice in Central Bohemia, Czech Republic (alt. 280 m, 7.1.2006).

Isolation and cultivation of the photobionts

Photobionts were isolated by the thallus fragment method (Ahmadjian, 1993) as follows: small fragments of lichen thalli (cross-sections of heteromerous thalli, soredia from *Lepraria*) were plated onto agar slants in Petri dishes (BBM 3N - Bold's basal mineral medium according to Deason and Bold (1960) with three times the original amount of nitrogen) and incubated at 18°C, under an illumination of 20–30 mmol m^{-2} s^{-1} and a 16:8 h light-dark cycle. If fungal contamination occurred during the cultivation, the contaminants were carefully removed or the thalli fragments were transferred to new plates. After 2–3 weeks, groups of dividing algal cells were observed associated with some of the fragments. To obtain unialgal cultures, small populations of photobionts were transferred to BBM 3N and *Trebouxia* medium (according to Ahmadjian 1993). The isolates were cultivated under the conditions mentioned above. Cultured strains are maintained in the culture collection of O. Peksa at the Department of Botany, Charles University in Prague (strain numbers are included in Table 2).

Microscopy

The pure algal samples and fragments of lichen thalli were examined under light microscopy with an Olympus BX 51 with differential interference contrast optics, and by laser scanning confocal microscopy with a Leica TCS SP2 equipped with an Argon-Krypton laser using a 488 nm excitation line and AOBS filter free system collecting emitted light between 498 and 700 nm. A Leica 63x/1.4 N.A. oil immersion or 63x/1.2 water immersion objective fitted on the Leica DM IRE2 inverted microscope was used. A series of optical sections of chloroplasts were captured and used for 3D reconstruction of their morphology. The autofluorescence of the chlorophyll was exploited for visualization of the chloroplast structure. For the final processing of the confocal images and visualization of various chloroplast structures, Leica Confocal Software, version 2.61 (Leica Microsystems HeidelbergGmbH) and the Image J 1.37v and 1.38t program (Abramoff et al., 2004) were used. Images were finally resampled for print by IrfanView 3.98 (Irfan Skiljan, Vienna University of Technology).

Observation scheme

Photobionts were observed A) within desiccated fragments of the lichen thalli; B) immediately after hydration of the fragments; C) within hydrated thalli (fresh thalli incubated 3 days in the same conditions as the isolates and cultures); D) during the process of their liberation from lichen thalli (at 3, 7, 11, 20, 30 and 40 days after plating thallus fragments onto agar).

DNA extraction, PCR, sequencing, BLAST search

Total genomic DNA was extracted from lyophilized cultures following standard CTAB protocols (Doyle and Doyle, 1987) with minor modifications. DNA was resuspended in sterile dH_2O and amplified by the polymerase chain reaction (PCR). Amplification of the ITS of nuclear rDNA was from an algal-specific primer nr-SSU-1780-5' (Piercey-Normore and DePriest, 2001) and a universal primer ITS4-3' (White et al., 1990). For the amplification of the actin type 1 locus, we used the combination of algal specific primers a-nuact1-0645-5' and a-nu-act1-0818-3' (Nelsen and Gargas, 2006). PCR amplifications were performed using Red Taq DNA Polymerase (Sigma), cycling conditions for the ITS rDNA follows Piercey-Normore (2006), for Actin 1 locus Nelsen and Gargas (2006). PCR products were quantified on 1% agarose gel and cleaned with Genomed Jetquick Kit. Sequencing PCR was performed using BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems), sequencing on an ABI 3100 Avant genetic analyzer sequencer in 50 cm long capillary. Homology between obtained sequences and those currently available in the NCBI database was assessed using the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, NIH, http://www.ncbi.nlm.nih.gov/BLAST; Altschul et al., 1997) score system.

3. Results and Discussion

Identity of photobionts

For our investigation of photobionts, we chose four morphologically and taxonomical varied lichen taxa collected at the same stand. Interestingly, we distinguished only two species of symbiotic algae within the chosen lichens. Based on morphological and biological characteristics (Table 1) as well as ITS rRNA and partial Actin 1 sequences, we identified *Asterochloris* sp. in thalli of *Cladonia foliacea* and *Lepraria* sp., and *Trebouxia incrustata* Ahmadjian ex Gärtner in *Lecidea fuscoatra* and *Xanthoparmelia conspersa* (photobionts of lichen taxa *C. foliacea*, *L. fuscoatra* and *X. conspersa* are being described here for the first time). Both pairs of photobiont strains were genetically highly similar. Sequence similarities of *Asterochloris* strains were 100.0% in ITS rDNA (528 total nucleotides / 0 different nucleotide positions) and 99.8% in the Actin type 1 locus (517/1). Similarly, ITS rDNA similarity between *Trebouxia* photobionts was 99.6% (707/3). The findings of only two photobiont strains in four diverse lichens with different predominant reproduction mode at one stand supports a theory of photobiont-sharing among several mycobionts at one locality as described by Beck et al. (2002).

We confirmed that identification of photobionts from the genera *Asterochloris* and *Trebouxia* on the basis of morphological characters is difficult, and molecular methods are advisable for accurate determination. Based on a combination of microscopic features, strains isolated from *C. foliacea* and *Lepraria* sp. resemble species belonging to *Asterochloris* s. str. in many respects. Of particular note was the parietal position of chloroplasts prior to cell division, observed in both investigated strains, that constitutes one of the main discriminative features of the genus (Ahmadjian, 1960; Hildreth and Ahmadjian, 1981; Friedl and Gärtner, 1988; Škaloud and Peksa, 2008). The spherical shape of the cells, chloroplast with incisions running closely to the naked pyrenoid and the great number of aplanospores (most often 64 in sporangium) closely fit the description of *Trebouxia excentrica* according to Gärtner (1985). However, a BLAST search for the ITS rRNA sequences of both strains listed only ≥97% homology with the authentic culture of *T. excentrica* UTEX 1714 (AF345433; Piercey-Normore and DePriest, 2001). By contrast, the same BLAST search listed one sequence sharing ≥99% homology with the query. This sequence was obtained from an unidentified strain of *Asterochloris* sp., isolated from lichen *Stereocaulon saxatile* (DQ229886; Nelsen and Gargas, 2006).

The strains from *L. fuscoatra* and *X. conspersa* have characteristics similar to *T. incrustata* and/or *T. jamesii*. They are distinguished by deeply lobate chloroplasts with fine lobes (crenulate) to larger oblong ridged lobes (resemble rips by *T. jamesii*) and naked pyrenoid. We also observed nonsynchronous divisions in autosporangia, as mentioned by Gärtner (1985) as a typical feature of *T. incrustata*. A BLAST search for the ITS rRNA sequences of both photobionts from *L. fuscoatra* and *X. conspersa* listed 18 sequences sharing ≥99% homology with the query. Among many sequences derived from uncultured and unidentified photobionts, a sequence of the authentic strain of *T. incrustata* UTEX 784 was displayed (AJ293795; Helms et al., 2001).

Chloroplast changes

Microscopic investigation of photobionts from both lichenized and cultured stages was carried out for determination of chloroplast morphology in different ecological and physiological conditions as well as ontogenetic stages. We observed distinct differences among various life stages of algal cells and confirmed an influence of different living conditions on chloroplast structure. Chloroplast morphology was predominantly dependent on physiological (dryness/dampness) and ecological status (lichenized vs. free-living) in both investigated algal species. Ontogenetic changes were obvious, especially in cultured (free-living) forms of photobionts.

In the lichenized algae, a chloroplast is usually modified in some way. Within a desiccated lichen thallus (herbarium specimen) it is highly deformed, and distinctly compressed within a dehydrated protoplast; the pyrenoid is small, and poorly visible in only a portion of cells (Fig. 1a). A similar effect was observed by De los Ríos et al. (1999) in desiccated thalli of *Lasallia hispanica* and *Parmelia omphalodes*. They described very collapsed, star-like shaped cells with a small pyrenoid full of pyrenoglobuli. However, such deformations of the chloroplast is an obvious result of physiological drought. Immediately after hydration of a dry thallus chloroplasts are very rapidly changing into globular compact forms without lobes or with poorly visible lobes (Fig. 1b). In this form, the pyrenoid is clearly observable including penetrating thylakoids. However, this rehydration is possible only in relatively fresh specimens. As demonstrated by Honegger (2003), protoplast of the algal cells in thalli stored several years at room temperature failed to rehydrate and appeared irregular in shape (chlorophyll's autoflorescence is also lost).

According to our experience with isolation of photobionts, their viability varies in different types of lichen thalli: it is shorter in homoiomerous (leprose) thalli than in heteromerous. Viability (possibility of isolation and cultivation) of photobionts from thalli of *Lepraria* spp.

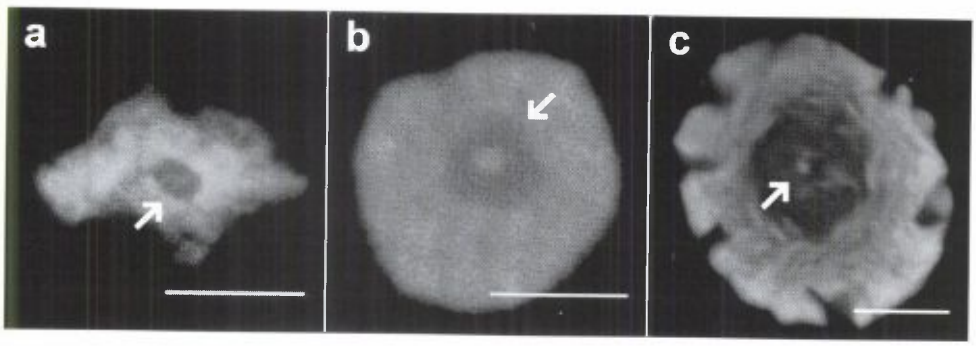

Figure 1. Effect of hydration to chloroplast structure of *Asterochloris* sp. from *Cladonia foliacea* – confocal sections of chloroplast. a) „star-like" shaped chloroplast within a dessicated thallus (pyrenoid – arrow); b) globular compact chloroplast immediately after hydration – pyrenoid penetrated by thylakoid (arrow); c) lobate chloroplast within a wet (living) lichen thallus – pyrenoid distinctly penetrated by thylakoids (arrow) and surrounded with a starch sheath. Scale bar: 5 μm.

stored at room temperature is likely up to 6 months at the most. By contrast, algae from lichens with heteromerous thalli are viable after storage for several years.

Physiologically active cells within a wet lichen thallus (after 3 days in wet conditions) have more developed chloroplast as compared with recently hydrated cells (Fig. 1c). The chloroplast occurs in several morphological stages with different degrees of lobation, but in comparison with cultured algae it is rather simple. Ahmadjian (1992) described changes of algal cells during resynthesis of the lichen thallus. The cells of the *Trebouxia* photobiont from *Acarospora fuscata* became smaller after they were enclosed in fungal pseudoparenchyma, chloroplasts enlarged and filled more of the cell, and the pyrenoid became larger and clearer. Our observations confirmed this pattern. Lichenized algae have more compact, less structured chloroplasts and the pyrenoid/chloroplast ratio is approximately double that of free-living algae. The mechanism of conversion from an intricate structure to a simple form is unclear, but it is evidently evoked by the mycobiont. The fungus can influence the alga either physically (direct contact, haustoria) or physiologically (chemically). During our investigation, we did not find an obvious relationship between physical contact of bionts and deformation of chloroplasts. For example, we observed very different haustoria types in *L. fuscoatra* and *X. conspersa* (deeply penetrating intracellular haustoria and shallow invaginated intraparietal haustoria, respectively), however, photobionts in both lichens had very similar changes in their chloroplasts. Deformation of chloroplasts is more likely to be caused by a physiological influence of the mycobiont unrelated to mechanical impact. However, very little is known to date concerning the chemical (hormonal) activity between the bionts.

In all studied photobionts, we further observed remarkable changes in the internal chloroplast structure, namely a fluctuation in the amount of starch. Jacobs and Ahmadjian (1971) described changes in starch volume that were dependent upon water content in the photobiont of *Cladonia cristatella*. In wet conditions algae produce starch which is deposited in chloroplasts, and then in dry periods the starch disappears from algal cells. In our confocal images, starch is clearly observable as a dark area around the pyrenoid, therefore we were able to observe its fluctuation in different stages of algal cells. After approximately 10 days of cultivation, the cells contained the highest volume of starch (the majority of the chloroplast is filled by a starch area around the pyrenoid). By contrast, the chloroplast in dry and immediately hydrated cells seems to form a compact mass without starch (compare Figs. 1a,b with Fig. 2b).

During the liberation of photobiont cells from the lichen thallus, the chloroplast changed significantly in its shape and structure (Figs. 2, 3). *Asterochloris* strains in particular underwent distinct changes. The process of liberation, when a new generation of algal cells is fully developed and liberated from a fungal hyphae, took about five weeks. We didn't observed any differences in behavior of related strains from different lichens (timing as well as morphological changes were almost identical). One of the remarkable chloroplast modifications takes place around the 10th day after the inoculation of a thallus fragments onto agar based medium (while still in the lichenized generation of algal cells). Chloroplasts of all cells in the population appear richly lobate and simultaneously contain a large volume of starch (Fig. 2b). During this initial period of being released from lichen thalli algae evidently adapt themselves to the new conditions of light and hydration (~ lag phase) and the decreasing influence of the mycobiont, which withers away due to unsuitable conditions (continuous moisture).

Approximately on the 20th day of cultivation, algal cells enter a phase of intensive division, characterized by the dominance of autospore (*Trebouxia*) or aplanospore (*Asterochloris*) packages (Figs. 2c, 3c). It is remarkable that during this phase algae maintain the mode of reproduction typical for their lichenized state: especially in *Trebouxia* we observed autosporangia with only 8 spores 20 days after the inoculation, however, aplanosporangia with 16–32 spores and zoosporangia with 64 spores did occur in mature cultures. The influence of the mycobiont evidently persists despite its considerable weakening under unsuitable conditions. Therefore, in parallel with chloroplast structure, the mode of reproduction noticeably changes during the formation of a new algal generation.

Mature cells of the new generation (30 and 40 days after the inoculation) were characterized by very different chloroplast shape compared with the lichenized generation. Moreover, several ontogenetic (morphological) stages of the chloroplast were observable in mature cultures (Figs. 2d, 3d). Each strain was characterized by either predominance or total absence of specific morphogenetic traits of the chloroplast (e.g. the photobiont of *C. foliacea* forms predominantly chloroplasts with unbranched lobes extended longitudinally at their ends – Fig. 2d; the strain from *L. fuscoatra* formed neither flattened nor extremely small fine lobes observed by strain from *X. conspersa* – Fig. 3d).

In addition to the investigation of morphological changes, it would be very interesting to observe physiological changes in photobionts. Green and Smith (1974) investigated physiological differences between lichen algae in symbiosis and following isolation. They found almost immediate changes in the physiology of algae arising after isolation from a lichen thallus (i.e. little ^{14}C release, high incorporation into ethanol-insoluble materials, low incorporation into simple carbohydrates). In contrast to the fragment method of isolation, they liberated algal cells from fungal hyphae very quickly through successive centrifugations of 30 to 60 minutes.

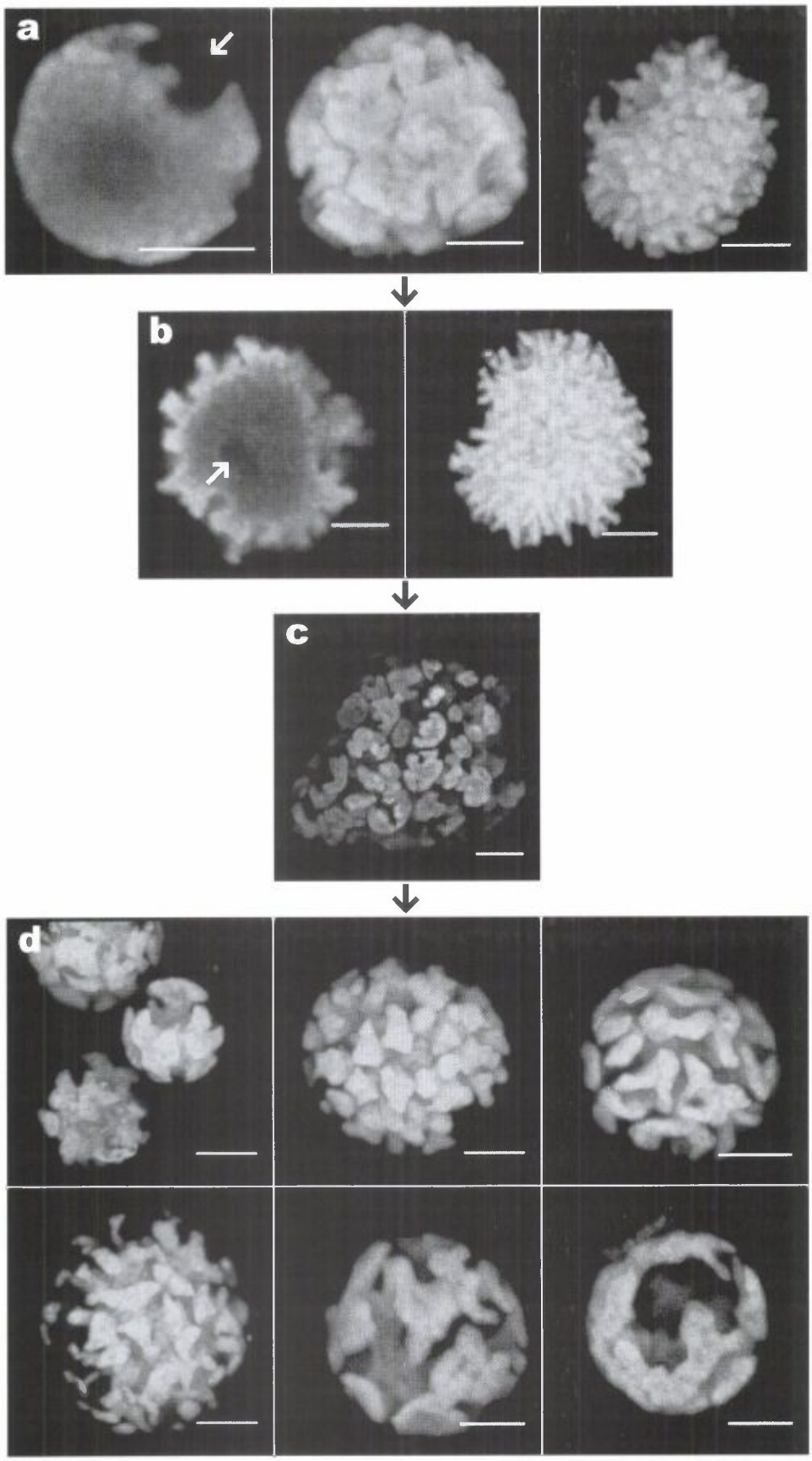

Figure 2. Chloroplast changes in *Asterochloris* sp. during the liberation from thalli of *Cladonia foliacea* (Clad) and *Lepraria* sp. (Lep) – confocal sections (first from the left in a, b) and maximum projections of chloroplast.
a) Different chloroplast types of lichenized photobionts within a wet lichen thallus – pyrenoid and invagination for nucleus (arrow) well observable (Lep);
b) strongly lobate chloroplast on the 7th day of cultivation – the chloroplast is filled by an extensive dark stained starch area around the black pyrenoid (arrow) (Lep, Clad);
c) phase of intensive division (20th day) – mature aplanopores (Lep);
d) fully liberated generation of photobionts 30–40 days after the inoculation – several ontogenetic stages of chloroplasts with different morphologies occuring in culture (from the top left): simple lobate form of young cells; crenulate form; chloroplast with unbranched lobes extended longitudinally at their ends (elongate appearence in surface view); fragile T-shape lobes; deeply incised, broadly extended lobes branched in terminal part; chloroplast in parietal position prior to cell division (all figs. Lep).
Scale bar: 5 μm.

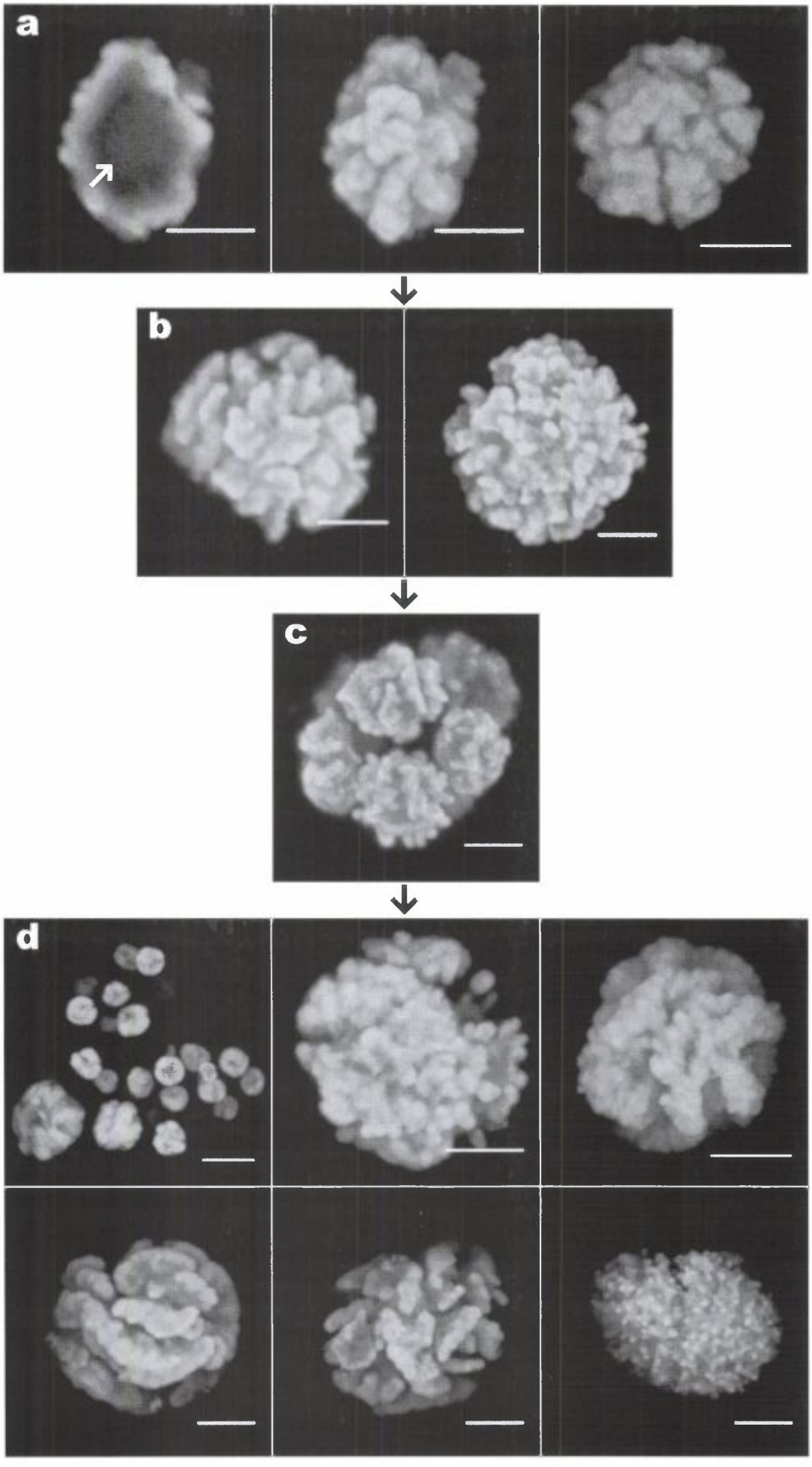

Figure 3. Chloroplast changes in *Trebouxia incrustata* during the liberation from lichen thalli of *Lecidea fuscoatra* (Lec) and *Xanthoparmelia conspersa* (Xant) – confocal sections (first from the left in a) and maximum projections of chloroplast.
a) Chloroplasts of lichenized photobionts within a wet lichen thallus – pyrenoid well observable (arrow) surrounded by a dark stained starch area (the first two Lec; Xant);
b) chloroplast 10 days after the inoculation (Xant);
c) phase of intensive division (20th day) – autospore package (Lec);
d) fully liberated generation of photobionts 30–40 days after inoculation – several ontogenetic stages of chloroplasts with different morphologies occuring in culture (from the top left): aplanospores and young cells (Lec); chloroplast with small rounded lobes (crenulate) (Lec); ridged lobes (2 types – Lec, Xant); flattened lobes (Xant); extremely small and fine lobes (Xant).
Scale bar: 5 μm.

However, when compared with the data of Richardson and Smith (1968), their results showed some residual effect of the mycobiont on the isolated algae (in contaminated or less washed preparations). Thus, the mycobionts' influence on algae probably decreases gradually when the fragment isolation method is used.

It would be advisable to investigate in detail the changes occurring in the chloroplast during the synthesis of a lichen thallus. When does the change from the lobate chloroplast of free-living algae to the simple lichenized form appear? It may already develop in the initial stage of contact between bionts (envelopment of the algal cells by fungal hyphae), but it seems more likely that it may arise during formation of the pseudoparenchymatic tissue in the first true lichenized unit.

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