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Studies on initiation and development of the partner association in *Geosiphon pyriforme* (Kütz.) v. Wettstein, a unique endocytobiotic system of a fungus (Glomales) and the cyanobacterium *Nostoc punctiforme* (Kütz.) Hariot

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Dedicated to Prof. Dr. Dr. h.c. Eberhard Schnepf on the occasion of his retirement

Summary. Phagocytosis of *Nostoc* filaments by *Geosiphon*, a fungus closely related to AM forming Glomales, was observed under light microscopes. Incorporation can only be performed if *Nostoc* primordia come into contact with growing hyphal tips of the fungus. The fungal wall just below the apex softens, and fungal cytoplasm is bulged out repeatedly covering the vegetative *Nostoc* cells but not the heterocytes. New heterocytes are differentiated by the internalised filament whose cells can increase up to ten times in volume after recovering from incorporation strain. The *Nostoc* cells are coated stepwise by short finger-shaped protuberances of the fungal hypha. These hernia-like outgrowths first remain separated, after 1 to 2 days they merge. Adjacent hyphal walls inside the complex covering disintegrate. Periphal fungal wall portions are united to form a smooth strong outer envelope. Internalisation is categorised as phagocytosis. The partnership is partly specific, *Nostoc* strains capable of living endocytobiotically are often partners in other symbioses besides *Geosiphon*.

Keywords: Cyanobacteria; Endocytobiosis; Endosymbiosis; *Geosiphon*; *Nostoc*; Phagocytosis.

Abbreviations: AM arbuscular mycorrhiza (formerly, VAM vesicular arbuscular mycorrhiza); DIC differential interference contrast; LD light/dark.

Introduction

Geosiphon pyriforme (Kütz.) v. Wettstein is a coenocytic soil fungus closely related to arbuscular mycorrhiza-forming fungi of the genus Glomus (Mollenhauer 1992; Kluge et al. 1994; Schüßler et al. 1994, 1995, 1996; Schüßler 1995). Up to now it is the only known example of a fungus living in endocytobiotic association with a photoautotrophic microsymbiont, i.e., with the cyanobacterium *Nostoc punctiforme* (Kütz.) Hariot. The present knowledge of *Geosiphon* has recently been reviewed (Kluge et al. 1994, Mollenhauer and Kluge 1994). Due to the physiological activities of the endocytobiotic *Nostoc* the symbiotic consortium exhibits carbon- and nitrogen-autotrophy (Kluge et al. 1991, 1992; Bilger et al. 1994).

Nostoc punctiforme and *Geosiphon* are found living together in the same habitat in and on the soil. Here, *Nostoc* undergoes a characteristic life cycle starting from akinetes that are transported by water seeping into the ground downward from the soil surface. Germinating akinetes give rise to hormogonia (motile trichomes; for details of terminology of the nostocacean life cycle, see Castenholz 1989, Komárek and Anagnostidis 1989, Bilger et al. 1994, Mollenhauer et al. 1994, Dodds et al. 1995) that perform positive phototactic movements in dim light and negative phototactic movements in strong light. The *Nostoc* hormogonia gather just below the soil surface, thereby undergoing transformation into "primordia" and develop further into gelatinous vegetative plant masses ("thalli").

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These in turn can be transformed into hormogonia or into akinetes. After formation of hormogonia, cells first enlarge and protrude laterally without dividing. Thus the longitudinal axis of the filament is folded. When cell-division is taken up again, new cross walls are arranged perpendicular to the filament axis and also at different angles. This aspect has often been mistaken for longitudinal division or even for separation of cells. They are said to rearrange into filaments later. However, there is no basis for such assumptions. Therefore Mollenhauer et al. (1994) consider the term "primordium" more appropriate. It refers to a new start beginning with processes of reorganisation including DNA synthesis, re-establishment of a regular pattern of cell growth and cell division, formation of a fresh gelatinous envelope. A primordium is a stage of its own. This is obvious as only primordia "infect" *Geosiphon* (see below).

The life cycle of the fungal partner of the *Geosiphon* consortium starts from white to yellowish-brown spores which also represent stages of perennation. The structure of the spores has been described elsewhere (Schüßler et al. 1994, Schüßler 1995). Soon after formation, spores germinate easily. In older spores germination is difficult to induce. Readiness to germinate increases after exposition of spores to cold or by addition of unidentified and nonspecific exudates from bryophytes. Higher temperature (25 °C) favours germination and growth of hyphae. This behaviour resembles that of many spores of AM fungi. A germinating spore of *Geosiphon* produces one hypha, it very rarely produces more than one. The germinating hypha soon ramifies to form an expanding mycelium. Hyphae grow horizontally at a depth of 1 mm inside the soil. Growth of hyphae from spores is limited by the nutrient supply from the storage products in the spore. Growth eventually stops, unless one or more young hyphae meet and incorporate *Nostoc* filaments in a process which will be described later in more detail. In order to distinguish a primary hypha that has been transformed into a *Geosiphon* bladder from those that grow out of this bladder and incorporate another cyanobacterium, we call the latter secondary hyphae.

Each incorporation event leads to the formation of a pear-shaped overground fungal bladder from 0.5 to 2 mm in length in which a *Nostoc* filament is enclosed. *Nostoc* cells then multiply and become physiologically active endosymbionts. The bladders appear between the uppermost particles of loamy moist soil from late spring until the first frosty days in late autumn.

A recent reinvestigation of the symbiotic consortium using improved techniques of electron microscopy and affinity techniques (Schüßler 1995, Schüßler et al. 1996) supplemented the results of Schnepf's (1964) pioneering study on ultrastructure. It was shown that the material located between the symbiosomal membrane and the outer membrane of *Nostoc* consists of compounds of the fungal cell wall. It is thus not produced by the cyanobacterium but deposed through a membrane homologous to the fungal plasmalemma.

In contrast the internal structure of the *Geosiphon* bladder, the crucial step in the early life history of the consortium, i.e., the incorporation of the cyanobacterial microsymbiont by the fungus, and the early stages of development of the bladder have not yet been described in detail. Without detailed knowledge of that step the correct interpretation of the unique association between *Geosiphon* and its cyanobacterial partner and its position within the general context of endocytobioses will be difficult. We have carried out light microscope studies on cultured material. Laboratory cultures of the *Geosiphon* were found to be very suitable for investigating mutual interactions of the symbiotic partners on diverse levels of integration (Kluge et al. 1994, Schüßler et al. 1995, Schüßler 1995).

Material and methods

Stock cultures of *Geosiphon* were initiated from material collected at the natural stands near Bieber (Spessart Mountains, Federal Republic of Germany) and were maintained under standard conditions (Mollenhauer et al. 1994) of FIS Culture Collection of Cyanobacteria and Algae, Phycological Section, Außenstelle Lochmühle of Forschungsinstitut Senckenberg (FIS). Stock cultures of *Nostoc* are maintained on agar in test tubes in a culture room at 15 °C and a 14 : 10 h LD cycle illuminated by fluorescent light. Photosynthetically active photon flux density (PPFD; 400–700 nm) was 15 µmol photons/$m^2 \cdot s$. The culture medium in this study was BG 11 (Rippka 1988).

Young but fully developed *Geosiphon* bladders were used for the observation of *Nostoc* incorporation by the fungus. Such bladders were at least 3 weeks old. A number of secondary hyphae had already grown out of them. All hyphae were amputated. This can be done without damaging the bladder by using fine ophthalmologic operation scissors. The purpose of this procedure is to induce formation of many new actively growing secondary hyphae. The bladder was transferred together with a droplet of *Nostoc punctiforme* inoculum (FIS strain 1:1-26 = strain PCC 9503, Institut Pasteur, Paris) onto fresh culture medium. After 1 to 2 days, new secondary hyphae had emerged from the bladder, and added *Nostoc* filaments had been mostly converted to hormogonia. In this state the bladders were transferred together with the *Nostoc* hormogonia by means of a

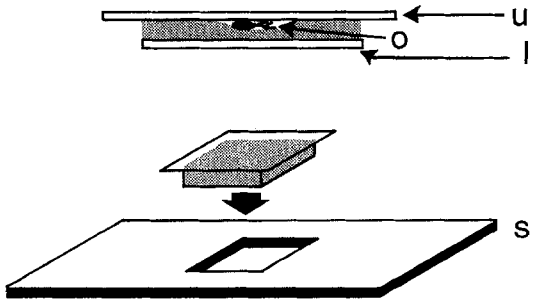


Fig. 1. Simple chamber for observation of living microorganisms (after Heunert 1973). Agar slant between two cover slips mounted on a slide and sealed with paraffin/vaseline. *l* Lower cover slip, *o* organisms, *s* slide (stainless steel), *u* upper cover slip

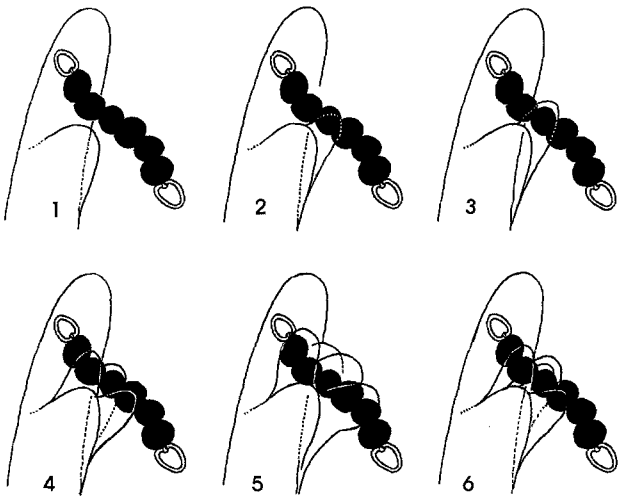


Fig. 2. Schematic survey of stepwise incorporation of a *Nostoc* filament (black, vegetative cells only) by *Geosiphon* cytoplasm protruding from a hypha

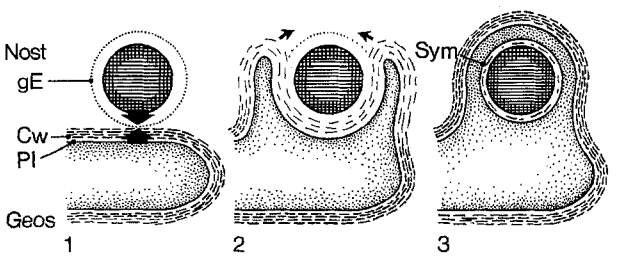


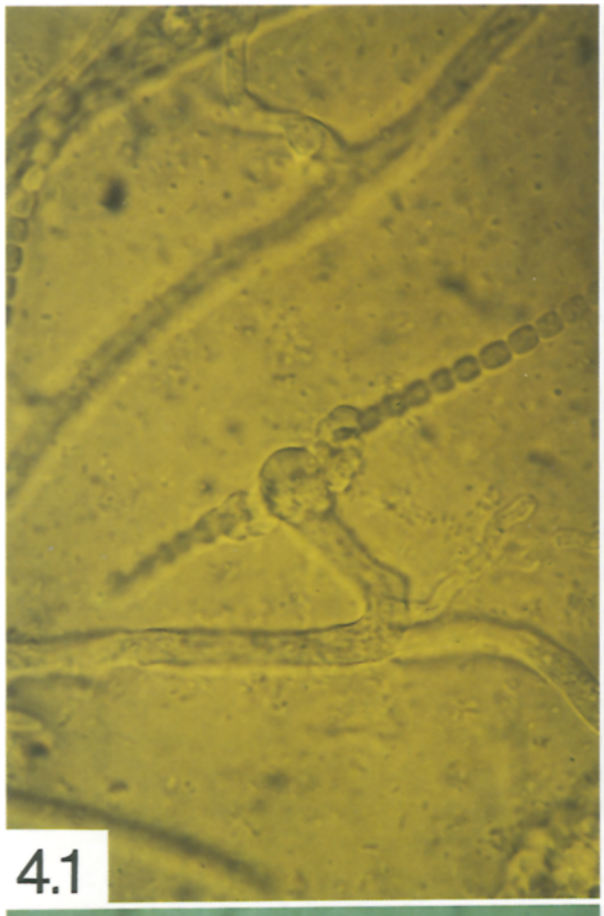
Fig. 3. Schematic survey of 3 stages of the same process as in Fig. 2. Hypha in longitudinal section, cyanobacterium in cross section. *Cw* Fungal cell wall, *gE* cyanobacterial gelatinous envelope, *Pl* fungal plasmalemma, *Sym* symbiosomal membrane

micro-spatule onto a sterilised piece of cellophane (20 mm × 20 mm) placed on the surface of fresh culture medium. The cellophane together with the underlying culture medium was put onto a sterile cover slip (22 mm × 22 mm), with *Geosiphon* on top (Fig. 1). The organisms and substrate plus an additional droplet of sterile nutrient solution (to avoid air bubbles), was then covered with a second sterilised cover slip (24 mm × 24 mm). The sandwich consisting of the two cover slips with the cellophane and the agar with organisms between them was mounted over a quadratic opening (22 mm × 22 mm) cut into the centre of a 3 mm thick microscope slide made of stainless steel (Fig. 1) (see also Heunert 1973). The margins of both the upper and lower coverslips were sealed with a mixture of equal amounts of paraffin and vaseline so that a water vapour-tight chamber with elastic sealing was formed. This chamber allowed continuous microscopic observation of the enclosed living organisms for days or even weeks with bright field optics. If Nomarski optics (DIC) or other microscopic techniques with polarised light were used the cellophane had to be omitted from the system shown in Fig. 1. Observations were made using various types of light microscopes (Leitz, Wetzlar; Zeiss, Jena; Zeiss, Oberkochen).

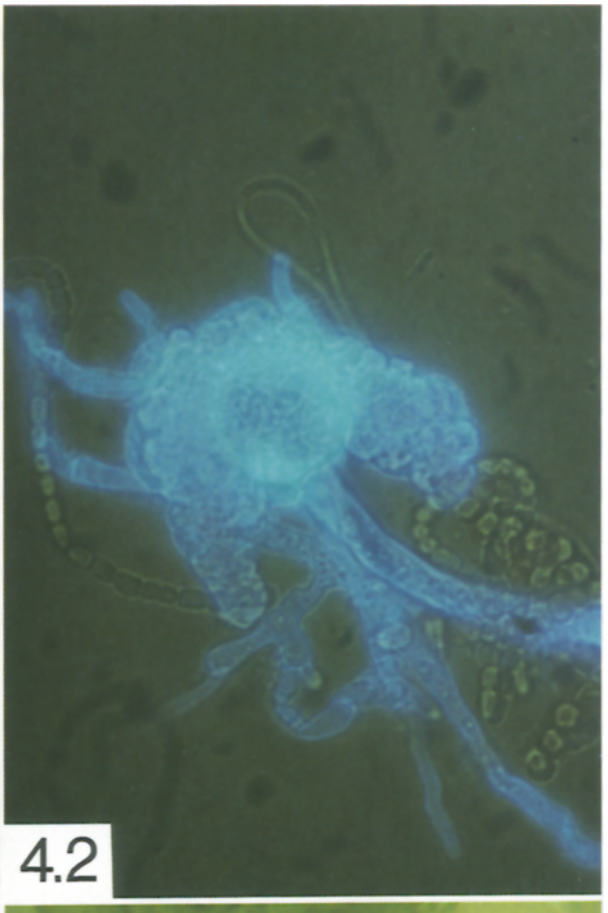
Results

In the laboratory, fresh hyphae easily emerge from actively growing *Geosiphon* bladders or from fungal spores that are ready to germinate. *Nostoc* can also be made to perform its developmental cycle in vitro. If the potential partners are brought together, incorporation of *Nostoc* by the fungus can be achieved and followed in detail by light-microscopy and microcinematography, provided both partners are in the appropriate developmental state.

The incorporation of *Nostoc* by the fungus is summarised schematically in Figs. 2 and 3 and shown in Fig. 4.1–2. Upon contact between a freshly formed *Nostoc* primordium and the apical region of an actively growing hypha a blunt projection of fungal cytoplasm abruptly bulges out just below the hyphal apex (Fig. 2.2). This process proceeds within a few minutes and resembles the emergence of pseudopodia in amoebas. Weak, but significant Calcofluor White fluorescence shows that the developing bulge remains to be covered by some fungal cell wall material (Fig. 4.2), most likely chitin (Schüßler et al. 1995, 1996). A few minutes after the first contact with the *Nostoc* cells, bulging of the hypha stops. The outer covering of the bulge gets thicker and more solid, and the intensity of its Calcofluor White fluorescence again increases. The whole process is then repeated several times within the direct vicinity of the primary bulge (Fig. 2.4–6). The hyphal tip first forms a dumbbell shaped (Fig. 4.1) and later a raspberry shaped mantle around the contacted *Nostoc* primordium. This mantle consists of densely packed separate portions of fungal plasma with an outer surface resembling pavement. In this stage the *Nostoc* cells are finally incorporated into the fungal coenocyte (Fig. 3.1–3). At the same time, the bulkhead-like compartmentation of the fungal extrusion disappears and its outer



4.1



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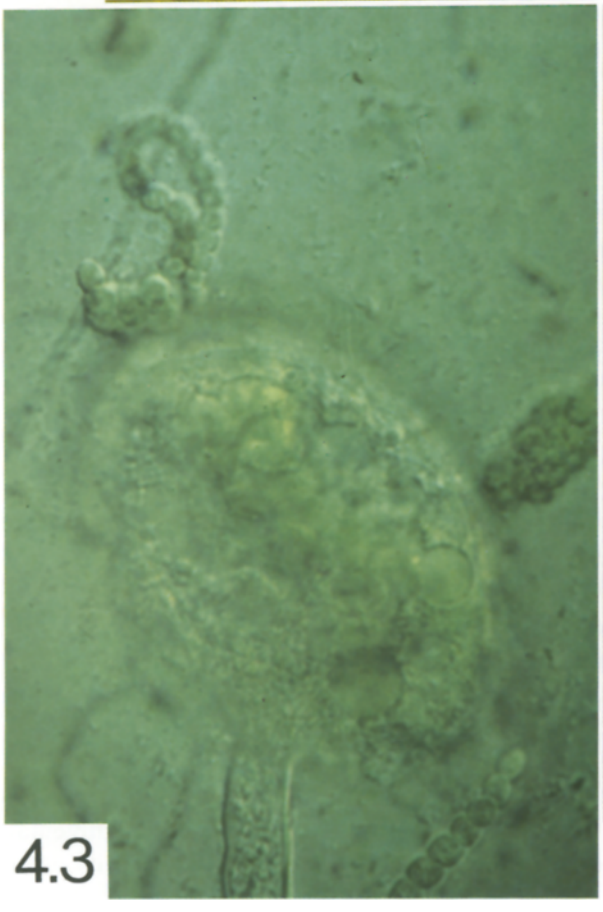

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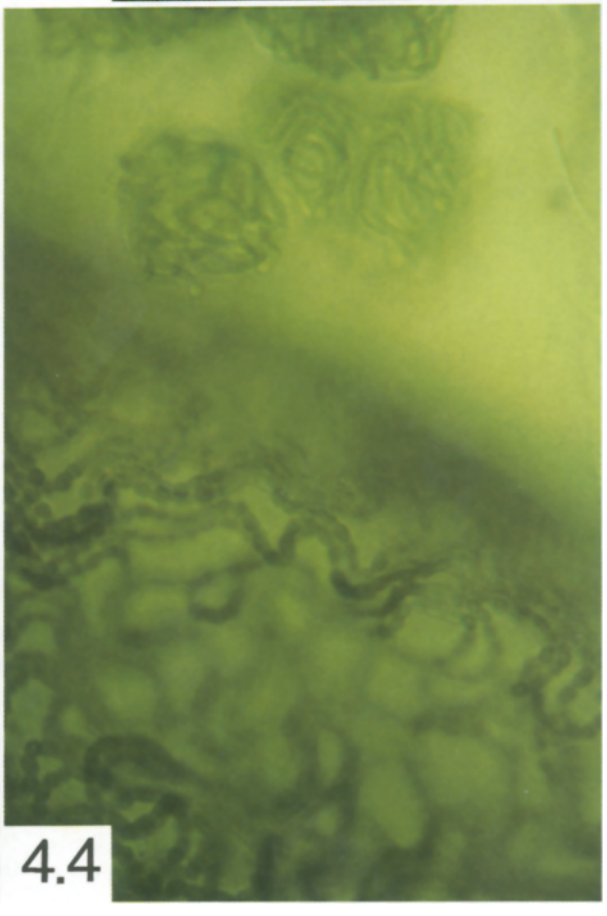

4.4

wall structure gradually becomes strengthened and smoothened. The newly formed bladder grows at its apical region. Cytoplasm containing many light-microscopically visible inclusions heavily falls into the young bladder through the primary hypha. Both hyphae protruded from germinating spores and those emerging from bladders are able to incorporate *Nostoc* in the described manner.

Nostoc cells continue to undergo typical development during and after their incorporation. If terminal heterocytes (primary heterocytes) are not yet differentiated when fungus and cyanobacterium meet, they can still be produced even if the primordium is already partially enclosed by the fungal protrusion. It is important to note that heterocytes are never mantled by the fungal cytoplasm and thus always remain outside the hypha.

The gradual alterations of the *Nostoc* cells occurring directly after incorporation are difficult to observe. In very young stages of development of a *Geosiphon* bladder, fungal cytoplasm surrounding the cyanobacterium is foamy and loses its transparency. Later during maturation of the bladder, the fungal cytoplasm becomes transparent again so that the endocytobiotic *Nostoc* cells can be directly observed.

There is no doubt that during the first 12 h after incorporation the *Nostoc* filaments are heavily impaired. Cell-to-cell connections weaken. Single cyanobacterial cells can be pushed out of the fungal bulge if pressure is exerted onto the cover slip. During the first 12 h after incorporation into the fungal hypha, the pigment contents of *Nostoc* cells is low and UV mediated autofluorescence of the photosynthetic pigments is significantly weaker.

During the following maturation of the *Geosiphon* bladder, the enclosed *Nostoc* filament recovers gradually from the strain. It becomes again visible and consists of more cells than upon incorporation. Cells now divide actively. Those which have developed inside the growing bladder have increased in volume by an order of magnitude when compared to free-living *Nostoc* cells (Fig. 4.4). Unpublished experiments conducted with our *Nostoc* strain 1:1-27 in Dr. F. Koenig's laboratory (University of Frankfurt a. M., Federal Republic of Germany) indicate that it is not osmotic pressure, but hydrostatic pressure that causes this cell enlargement (pers. comm.). Except for their larger size, the *Nostoc* cells resemble those outside the bladder. They are arranged in long filaments showing intercalary growth in which heterocytes are differentiated with the same regular distribution pattern (same frequency) as in filaments outside the fungal bladder. Also *Nostoc* cells are pushed towards the apex of the bladder by the flow-in of fungal plasma from the mycelium. The pigmentation of the recovered endosymbiotic vegetative *Nostoc* cells is restored and finally appears to be even more intense than that of free-living cells.

Throughout the entire process leading to the establishment of the symbiotic consortium *Geosiphon* the following steps can be distinguished:

1. Contact of the partners, initiation and completion of the endocytotic process (duration about 24 h).
2. Recovery of the incorporated cyanobacteria from incorporation strain (2–3 days).
3. Maturation of the *Geosiphon* bladder to the state when new hyphae can be extruded (about 3 weeks).
4. Maintenance of the functional bladder (about 6 months).

Discussion

The early steps in the establishment of the symbiotic consortium *Geosiphon* raise two major questions. One concerns the mechanism leading to the incorporation of the photobiont into the coenocytic fungal cell. The other question concerns the quality of the interaction between the partners including specificity and mechanisms of partner recognition. This second aspect needs further study.

Our experience with *Geosiphon* bladders and hyphae can be summarised by interpreting the growing coenocyte of *Geosiphon* as a continuous hydraulic system comparable to a plant cell which has to constantly maintain its turgor pressure. A damaged bladder cannot be repaired. If the wall breaks, a tangled cyanobacterial filament is pushed out. Phycocyanin soon leaks into the medium. The cells die immediately. Hyphae are like tubes with tensionproof walls containing a fluid under pressure. Local pressing on a

Fig. 4. Photomicrographs. **1** Young stages of incorporation of *Nostoc*, apex of fungal hypha transformed into dumb-bell shaped structure; DIC. **2** Later stage of incorporation, wall around protruded fungal cytoplasm softer (fluorescence not so distinct as that of differentiated hyphae); UV light, Calcofluor White staining. **3** Young bladder during initial phases of incorporation, *Nostoc* filaments containing low amounts of photosynthetic pigments. **4** Fully developed bladder; left, view into the bladder; right, small *Nostoc* thalli of same strain that developed outside the fungus. Cyanobacteria inside the bladder are larger than free living ones (cell diameter of free living *Nostoc* about 5 μm)

hypha pushes the cytoplasm aside. Damage of hyphae can be repaired. Inactive sections of the mycelium (e.g., older hyphae without contact to cyanobacteria) are partitioned off by bulkhead-like structures. If secondary hyphae are cut in distal areas (i.e., far off the bladder) the coenocytic system is again closed by formation of a partitioning wall. Cutting of a hypha in proximal areas, i.e., near the bladder, causes collapse of the hypha and sudden pressure drop inside the bladder. In this case, a centripetally ingrowing cross wall thickening closes the narrow outlet of the bladder. So the bladder itself is not impaired. During active growth of the hypha this outlet for the bladder remains open, especially during differentiation of a young bladder. Opening is maintained if turgor pressure is equal inside the bladder and inside the protruding hypha. The ring shaped inner thickening at the hyphal basis is the pre-formed site where the fully developed daughter bladder is to be separated from the hyphal system of the mother bladder. In all coenocytic organisms, softening of wall areas causes bulging. In *Geosiphon*, rare cases of excessive wall softening could be observed. Under these circumstances, fungal cytoplasm was leaking out after contact between hyphal tip and *Nostoc* primordium. Vital fungal cytoplasm always is covered by wall material whose consistency and solidity seems to be different depending in different ontogenetic phases. Neither we nor our coworkers (Schüßler and Steinmetz pers. comm.) ever observed naked fungal cytoplasm.

The uptake of endocytobionts by a type of closed hydraulic system can be envisioned as passage from low hydrostatic pressure through a lock chamber to high hydrostatic pressure. The initial phases of incorporation may be interpreted as the stepwise construction of a temporary lock chamber. First there is an outer opening. This is shut by the confluent fungal wall. The cytoplasmic interior is built up by separate compartments, resulting from the stepwise advancing process of coating the cyanobacterial filament. When the outer door of the lock chamber is shut bulkhead-like partition walls are no longer necessary. They now disintegrate, i.e., the inner door of the lock chamber is opened. Inside the transformed fungal hypha, *Geosiphon* cell wall material thus is in direct contact with the outermost layers of vegetative *Nostoc* cells. During this process, the gelatinous coating of the cyanobacteria disappears. So far, we do not know how.

We assume that in *Geosiphon* bulging of coenocytic cell contents just below the hyphal tip is brought about by the same mechanism as in all other fungi of this type. Protrusion of the cell surface results from internal hydrostatic pressure (turgor) at sites where the cell wall is locally softened. Turgor in vacuolated cells and coenocytic hyphae of *Geosiphon* is about 0.6 MPa (Schüßler et al. 1995). Their mature walls show a multilayered quasi-crystalline ultrastructure and are therefore quite tensionproof (Gooday 1995). Hyphae grow subapically by insertion of a ring of freshly synthesised cell wall material just below a protecting cap covering the hyphal tip. In this annular area of growth the immature cell wall is not crystalline, hence plastic (Gooday 1995) and thus vulnerable towards mechanical deformation due to cell turgor. Such deformation is the initial step of hyphal branching (Gooday 1995). Occasionally cytoplasm can also be observed to protrude "spontaneously" just below the hyphal tip without branching thus indicating damage (Jelke and Kreisel 1993). It is vitally important for coenocytic fungi and other coenocytic organisms to have mechanisms for repairing such damages of the outer envelopes. Repair mechanisms similar to *Geosiphon* are known to operate in coenocytic algae, i.e., in Dasycladales where plug like structures separate fertile branches during reproduction (Burr and West 1970), and in Vaucheriales upon attack by gall inducing rotifers (Rieth 1980). We have observed that in *Geosiphon* contact of a fully differentiated hypha with a young and actively growing tip of a second hypha causes the bulging of cytoplasm and initiation of repairs. Thus an anastomosis is formed between the two hyphae (D. and R. Mollenhauer unpubl.).

The process of incorporation of *Nostoc* into the fungal hypha needs to be studied at the ultrastructure level. Nevertheless, our present findings allow certain conclusions. There is little doubt that the incorporation proceeds by endocytosis or more precisely by phagocytosis (for terminology, cf. Kleinig and Sitte 1986). This process requires vesiculation of the plasmalemma to include the *Nostoc* primordium and to transport it into the interior of the fungal cell. Indeed, Schüßler et al. (1996) recently demonstrated using fluorescent studies of lectin labelled sugar compounds that inside the *Geosiphon* bladder the *Nostoc* cells remain enclosed in a plasmalemma-derived membrane. This membrane has retained the capability to synthesise chitin thus forming an envelope around the *Nostoc* cells inside the fungal bladder which is homologous to the fungal cell wall. In Fig. 3.3, this is indicated by the same signature between symbiosome membrane

and *Nostoc* cells as for the fungal cell wall. Further support for categorising the endocytosis of *Nostoc* in *Geosiphon* as phagocytosis is derived from unpublished findings (1991) by I. Steinmetz. She found cytoskeleton elements in the fungal hypha that become visible only during the process and near the site of *Nostoc* incorporation. The interface between the phagocytotically internalised cyanobacterium and *Geosiphon* is homologous to the interface between the outer membrane of arbuscules of AM fungi and the plasmalemma of root cells (Schüßler et al. 1996). Phagocytotic uptake of *Nostoc* would require, at least initially, free exposition of the fungal plasmalemma towards the future endosymbiont thus allowing vesiculation and enclosure of the cyanobacterial cells. Our finding that the site of uptake, i.e., the plasmatic bulge protruding from the hyphal tip, remains covered by some fungal cell wall material, is not contradictory to the assumption that *Nostoc* is taken up by vesiculation of the plasmalemma. The weak intensity of Calcofluor White fluorescence visible at the outer border of the bulge in the early stage of incorporation suggests that the amount of chitin present there is low. The chitin fibrils are not arranged in a crystalline structure as is the case in the rigid parts of the hyphal wall (Gooday 1995) but form a loose network (Schüßler unpubl.). It is difficult to show, though, it is very likely, that during the entire internalisation process fungal plasmalemma and prospective symbiosome membrane can remain interconnected and membrane flux is possible.

Acknowledgements

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