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Artificial Syncyanoses: the Potential for Modeling and Analysis of Natural Symbioses

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Abstract

New functional and structural features of artificial associations of cultured cells, tissues and whole plants with cyanobacteria were discovered. New data presented in this paper as well as earlier results, have been analyzed. The following acquisitions of artificial associations are revealed: the associations are capable of growing without or with low content of organic carbon or combined nitrogen; the frequency of heterocysts and nitrogenase activity of the cyanobiont increases; alkaloids are accumulated by *Solanum laciniatum* cells in association with nitrogen-fixing cyanobacteria in the absence of combined nitrogen; a new specific anatomical structure is formed; mucilage mantle is formed where the cyanobionts are localized; heteromorphic forms of cyanobacteria emerge in long-term-cultivated associations; symbiotic cyanobacteria are morphologically remodified in artificial association; a cyanobiont (*Nostoc muscorum* VKM 16) becomes obligately dependent on the plant partner. Comparison of artificial and natural syncyanoses shows that most newly acquired features of the artificial associations are similar to those of natural syncyanoses. The following applications of the method of artificial associations in symbiology are suggested: (1) *in vitro* modeling and investigations of symbiotic interactions on the cellular and tissue levels; and (2) maintaining *in vitro* of the symbiotic status of an isolated symbiotroph.

Keywords: cyanobacteria, artificial associations, modeling of natural symbiosis

Abbreviations: 2,4-D: 2,4-dichlorphenoxyacetic acid

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1. Introduction

Symbiosis is difficult to study in intact, native systems. Therefore there is a clear need for new and innovative approaches in the study of symbiotic systems (Taylor, 1983). Analysis of experimental symbiosis studies over the last 15 years has suggested that most studies can be classified into two types, reconstitution of a natural and formation of an artificial symbiosis.

The former approach calls for separation of natural symbioses and their resynthesis with the original partners or replacement of one partner by another (symbiotic or free-living). Such experiments have been carried out with symbioses of algae with *Paramecium* (Reisser, 1987), foraminifera (Lee et al., 1983), *Hydra* (Rahat, 1985), the flatworm *Convoluta* (Doonan et al., 1980), fungi (lichen resynthesis) (Ahmadjian, Jacobs, 1983; Galun and Bubrick, 1984; Galun, 1988) and of cyanobacteria with higher plants (syncyanoses reconstitution) (Silvester and McNamara, 1976; Rodgers and Stewart, 1977; Bonnett and Silvester, 1981; Enderlin and Meeks, 1983; Lin et al., 1988; Meeks et al., 1988; Kimura and Nakano, 1990; Meeks, 1990; Johansson and Bergman, 1991; Meeks and Campbell, 1991) and others. Because certain kinds of experimental symbioses have been described in a number of papers we refer only to reviews or to most recent papers.

In the latter approach, creation of artificial associations, organisms are used in such combinations which are not found in nature. Multicellular organisms are first "disassembled" so as to obtain cultures of isolated organs, tissues, cells or protoplasts. Unicellular organisms are used as cells or protoplasts.

Numerous artificial associations of quite different kinds of units have been reported. The plants (Patriquin et al., 1983; Vose, 1983; Gusev et al., 1986; Kerby et al., 1991; Gantar et al., 1991a,b; Okon, 1991) and isolated roots (O'Hara et al., 1983) of monocotyledonous and dicotyledonous plants were inoculated with free-living nitrogen-fixing bacteria. Non-legume plants were inoculated with rhizobia for nodulation of their roots (Cocking et al., 1991; Ridge et al. 1991). Higher plant tissue cultures were used in order to obtain associations with microorganisms (reviews by Vasil et al., 1980; Gusev et al., 1984; Kovacheva et al., 1986). Associations of invertebrate tissue cultures with green algae and dinoflagellates have been obtained (Taylor, 1978). Cells (Nicolov et al., 1983; Gyurján et al., 1986; Korányi et al., 1990) and mutants without cell walls of green algae (Nicolov et al., 1983) were grown in joint cultures with nitrogen-fixing bacteria.

Cells of green algae were used in associations with actinomycetes (Likhacheva et al., 1987). Cyanobacteria and algal organelles, cyanelles, were introduced into plant cells (Bradley, 1979), and green algae, into amoebae

cells (Jeon and Lorch, 1982). Intracellular symbiosis between an amoeba and bacteria that used to be pathogenic for the amoeba occurred spontaneously in the laboratory (Jeon, 1972). Finally, various species of microorganisms and their organelles were introduced into isolated higher plant protoplasts (reviews by Fowke and Gamborg, 1980; Giles and Vasil, 1980; Gamborg and Bottino, 1981; Gusev et al., 1984; Gusev and Korzhenevskaya, 1990), and nitrogen-fixing bacteria, into cell wall-less mutants of green algae (Nghia et al., 1986).

In our experiments with artificial associations we have inoculated different plant derived material such as isolated protoplasts, isolated cells, tissues, root cultures,plant-regenerates, plants propagated by cuttings and whole plants,with cyanobacteria. Our findings on the techniques for obtaining artificial associations and their characteristics have been published elsewhere (Gusev et al., 1984; Korzhenevskaya et al., 1986; Gusev and Korzhenevskaya, 1990). Only some of the associative systems obtained fit the criteria of symbiosis (Lewis, 1973; Starr, 1975; Margulis, 1981; Ahmadjian and Paracer, 1986; Smith and Douglas, 1987). In analogy withy natural associations of plants and cyanobacteria (Reisser, 1984) they were termed syncyanoses.

One of the characteristics of symbiosis is the new functions and structures which are not to be found in the original organisms. The objectives of this research were (1) to determine the new functional and structural features of the artificial associations; (2) to compare the functional and structural properties of artificial and natural syncyanoses; and (3) to evaluate applicability of artificial syncyanoses to modeling and studying natural symbioses.

2. Materials and Methods

Plant material and culture

1. *Panax ginseng* C.A. Mey cell culture, strain IPhR-G1 was grown aseptically on a modified Murashige and Skoog (MS) (1962) medium as previously described (Butenko et al., 1982).
2. *Medicago sativa* L. callus culture and plants propagated by cuttings were grown on B5 medium (Gamborg et al., 1968) to which various organic substances were added. A primary callus culture was obtained on B5(1) medium with (mg l^{-1}): myoinositol – 100, nicotinic acid – 1, pyridoxine – 1, thiamin – 10, 2,4-dichlorphenoxyacetic acid – 3, α-naphthyleneacetic acid – 0.5, kinetic – 8 and 30 g l^{-1} sucrose. In B5(2) medium for growth of transferred callus, 2,4-D and kinetin concentrations were reduced up to 0.1 mg l^{-1}. In B5(3) medium for induction of organogenesis the hormones were completely eliminated. B5(4) medium for plant regeneration

contained only 1 mg l^{-1} thiamin and 5 g l^{-1} sucrose. Plant cuttings were grown in B5(5) medium with 5 g l^{-1} sucrose, half the concentration of mineral salts and no vitamins and hormones. In some experiments the mineral nitrogen was eliminated, potassium ion deficiency was compensated by addition of 50 mg l^{-1} KCl. The media were solidified with 7 g l^{-1} agar, pH 5.75.

3. An *Oryza sativa* L. (cv. Kuban 3) callus culture was grown in a medium with MS mineral salts and addition of(mg l^{-1}): myoinositol – 80, yeast extract – 500, nicotinic acid, pyridoxine and thiamin – 1,6-benzylaminopurine – 4, kinetin – 0.1, 30 g l^{-1} sucrose, 7 g l^{-1} agar, pH 5.6–5.8.

Cyanobacteria cultures

Axenic cultures of cyanobacteria included: *Anabaena variabilis* Kütz. ATCC 29413, *Chlorogloeopsis fritschii* Mitra ATCC 27193, *Nostoc muscorum* Agardh. VKM 16 and *Nostoc* sp. isolated from the liverwort *Blasia pusilla* L. collected in nature (Zvenigorod Biological Station of Moscow State University). The liverwort thallus surface was sterilized with 30% H_2O_2 for 10 to 40 min. Then cyanobacteria colonies from the liverwort thallus cavities were transferred in a sterile syringe onto an agar medium. Simultaneously, the material was tested for heterotrophic contamination.

Suspension cultures of *A. variabilis*, *C. fritschii* and *N. muscorum* were grown in nitrogenous BG-11 medium (Stanier et al., 1971) or in nitrogen-free Allen and Arnon's (AA) (1955) medium. *Nostoc* sp. (the isolate from *B. pusilla*) was cultivated in BG-11 medium minus combined nitrogen with addition of 0.5% (w/v) fructose.

Pure cyanobacteria, plant cell, tissue cultures and noninoculated cuttings and plants are referred to as monocultures.

Obtaining of artificial syncyanoses

1. The method of obtaining and cultivating a mixed ginseng cell and *C. fritschii* culture was as previously described by Butenko et al. (1982).
2. Syncyanoses of alfalfa with *A. variabilis* and *N. muscorum* were obtained by inoculation of the plant material with 0.5–1 ml of two- or four-week-old cyanobacterial cultures having a density of 9×10^6 to 15×10^6cells ml^{-1}.

Inoculation was carried out by different methods.

Variants 1A and 1N

Leaf explants of aseptic alfalfa plants were placed on the surface of 5B(1) medium in glass beakers sealed with foil. *A. variabilis* (var. 1A) or *N. muscorum* (var. 1N), grown for 5–7 days in BG-11 medium, were then placed on the surface of explants and medium. Mixed calli, formed after a month, were transferred once in 1–1.5 months on the medium B5(2) or on B5(3), B5(4) and B5(5) media successively.

Variant 2A

A. variabilis culture grown in the AA medium was then placed on the callus surface at the moment of transfer of the callus from B5(3) to B5(4) medium. The callus had differentiated shoot-like structures which had been formed during cultivation on B5(3) medium.

Variant 3N

The stem cuttings of one-month-old plants with one axillary bud were placed on B5(5) medium. *N. muscorum* culture, grown in BG-11 medium, was placed on the medium surface in the immediate vicinity of the cutting. The cuttings rooted and the new shoots developed from axillary buds within 1 to 1.5 months.

Variant 4A

Cuttings fully rooted on B5(5) were transferred to a nitrogen-free B5(5) medium and were inoculated with *A. variabilis*, previously grown in AA medium, by applying the cyanobacteria to the root and medium surfaces.

3. Mixed cultures of *O. sativa* callus and *Nostoc* sp. (the isolate from *B. pusilla*) were obtained by inoculation of calli after their transfer to fresh medium with subsequent cultivating for 2.5 weeks. A 0.5 ml aliquot of a four-week-old cyanobacterial culture, at a density of 9×10^6cell ml^{-1}, was applied to the surface of both callus and medium.

In all experiments inoculated calli, cuttings and plants were incubated at 26±1° C, in continuous light (750–1200 lux).

Heterocyst frequency

Heterocyst frequency was calculated on the basis of their morphology by comparison with the vegetative cells. Heterocyst frequency is expressed as a percentage of the total cell number, at least 1000 cells were counted, and are the mean of three replicates.

Nitrogenase activity assay

The nitrogenase activity was estimated by the acetylene-reduction technique (Hardy et al., 1968).

Electron microscopy

Cyanobacteria and plant cells and tissues were fixed in 0.5% glutaraldehyde in Millonig buffer (pH 7.4) (Millonig, 1961) for 30 min at room temperature. Postfixing was in 1% OsO_4 in the same buffer for 4 hr at room temperature. The samples were dehydrated by passage through a graded ethanol series and embedded in araldite. Ultrathin sections were made with a LKB-8800 ultratome. Sections mounted on copper grids were stained with a basic lead citrate (Reynolds, 1963) for 40 min and were examined with a Jeol (JEM-100B) electron microscope. For determination of acid polysaccharides, samples were stained during fixation with ruthenium red (Luft, 1968). For scanning electron microscopy the samples were fixed and dehydrated by the above techniques. Dehydrated samples were placed in absolute acetone, left overnight and then critically-point-dried (Dryer HCP-2),coated with the Au-Pd alloy (IB-3 Ion Coater) and examined with Hitachi (S-405A) scanning electron microscope.

3. Results

Anatomical organization of artificial syncyanoses

As artificial syncyanoses emerge, the partners integrate morphologically and form mixed aggregates of plant cells and cyanobacteria in suspension cultures. Figure 1 shows a mixed aggregate of ginseng cells and *C. fritschii*. The cyanobacteria at first settle on the surface of aggregated plant cells and between them and also penetrate deep into the aggregates. Mixed alfalfa calli with *A. variabilis* (var. 1A) or *N. muscorum* (var. 1N) are organized in a similar way (Fig. 2).

The most complicated anatomical organization is characteristic of alfalfa plants infected with *N. muscorum* (var. 3N). The cyanobacteria occupy the surface of the stem base and the first to third leaves (Fig. 3). Also within the leaf mesophyll cyanobacteria may grow as microcolonies between plant cells. A microcolony is a compact group of encapsulated cyanobacteria, densely packed inside a shell made by the walls of degrading plant cells (Fig. 4). Degradation occurs only in the plant cells adjacent to the microcolonies. As microcolonies grow, plant cells that have lost their cytoplasm are compressed, and so the intercellular spaces expand (Fig. 4). This restructuring of the plant tissues results in the formation of "harbors" for cyanobacterial microcolonies. As a rule,

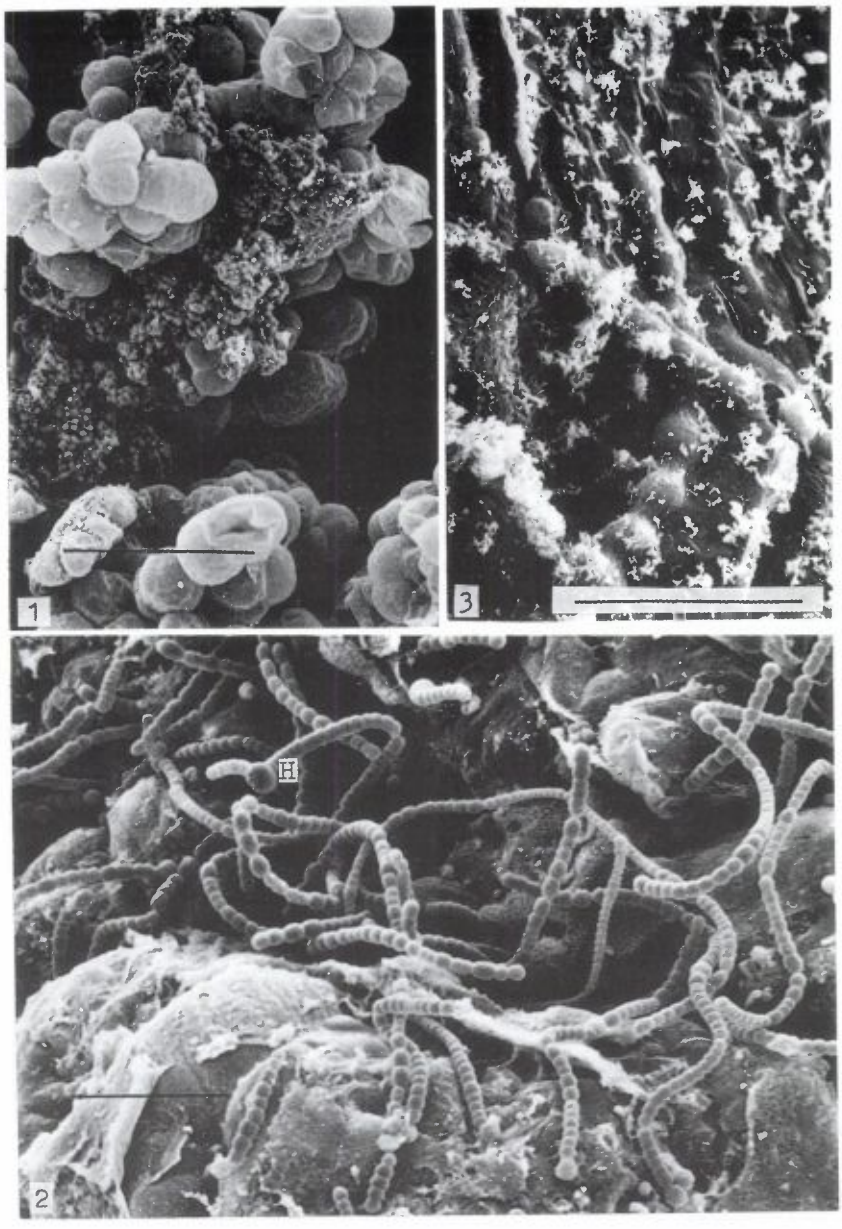


Figure 1. Mixed aggregate of ginseng cells and *C. fritschii*; joint cultivation for 14 d (bar = 200 μm).

Figure 2. Mixed alfalfa callus and *A. variabilis* (var. 1A); joint cultivation for 17 d. H, heterocyst (bar = 60 μm).

Figure 3. *N. muscorum* on the leaf surface of the 1.5–month-old alfalfa plant (var. 3N) (bar = 20 μm).

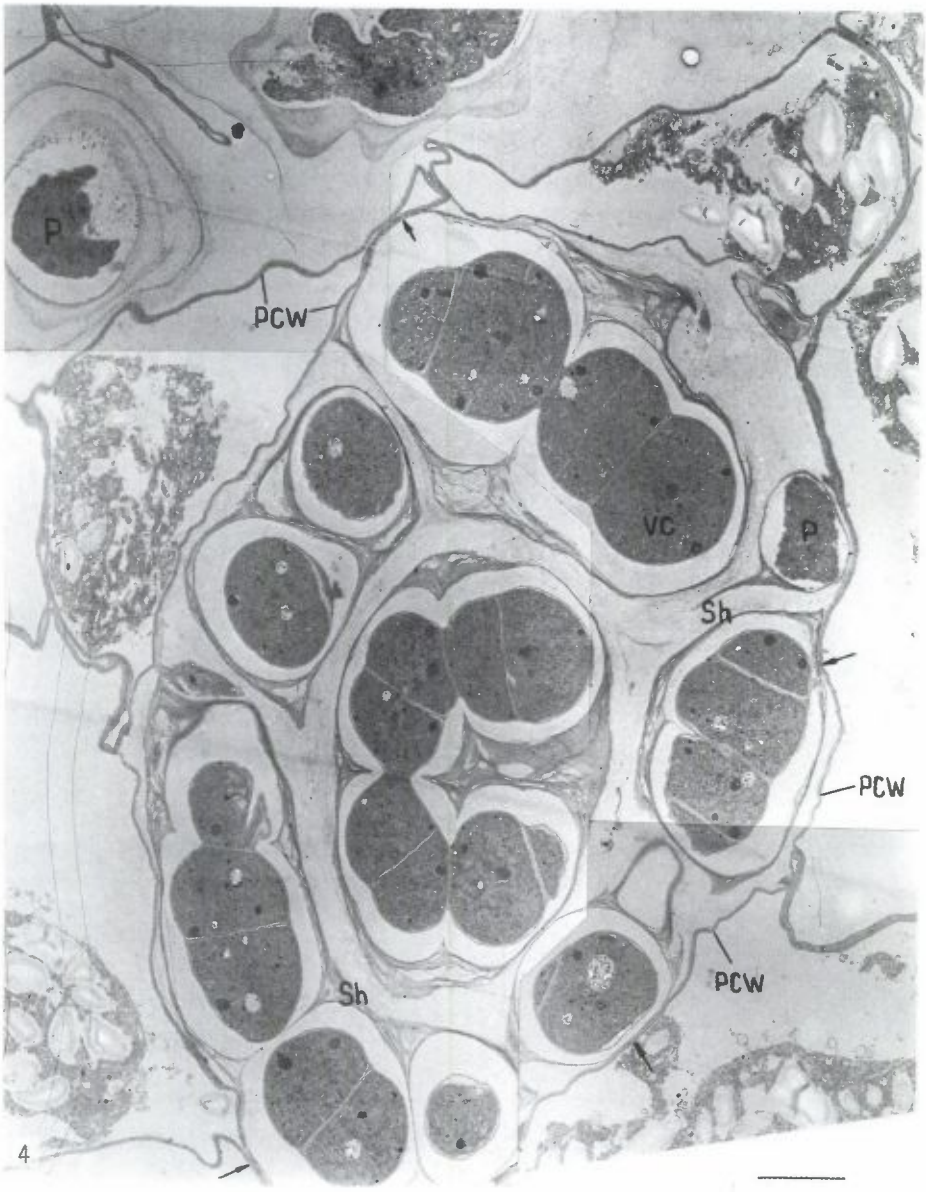


Figure 4. The microcolony of *N. muscorum* inside a shell made by the walls of degrading plant cells of alfalfa leaf mesophyll (var. 3N, the same plant as in Fig. 3). VC, cyanobacterial vegetative cell; P, cyanobacterial protoplast; Sh, sheath; PCW, plant cell wall surrounding the microcolony; arrows – the sites of fusion of PCW and Sh (bar = 1 μm).

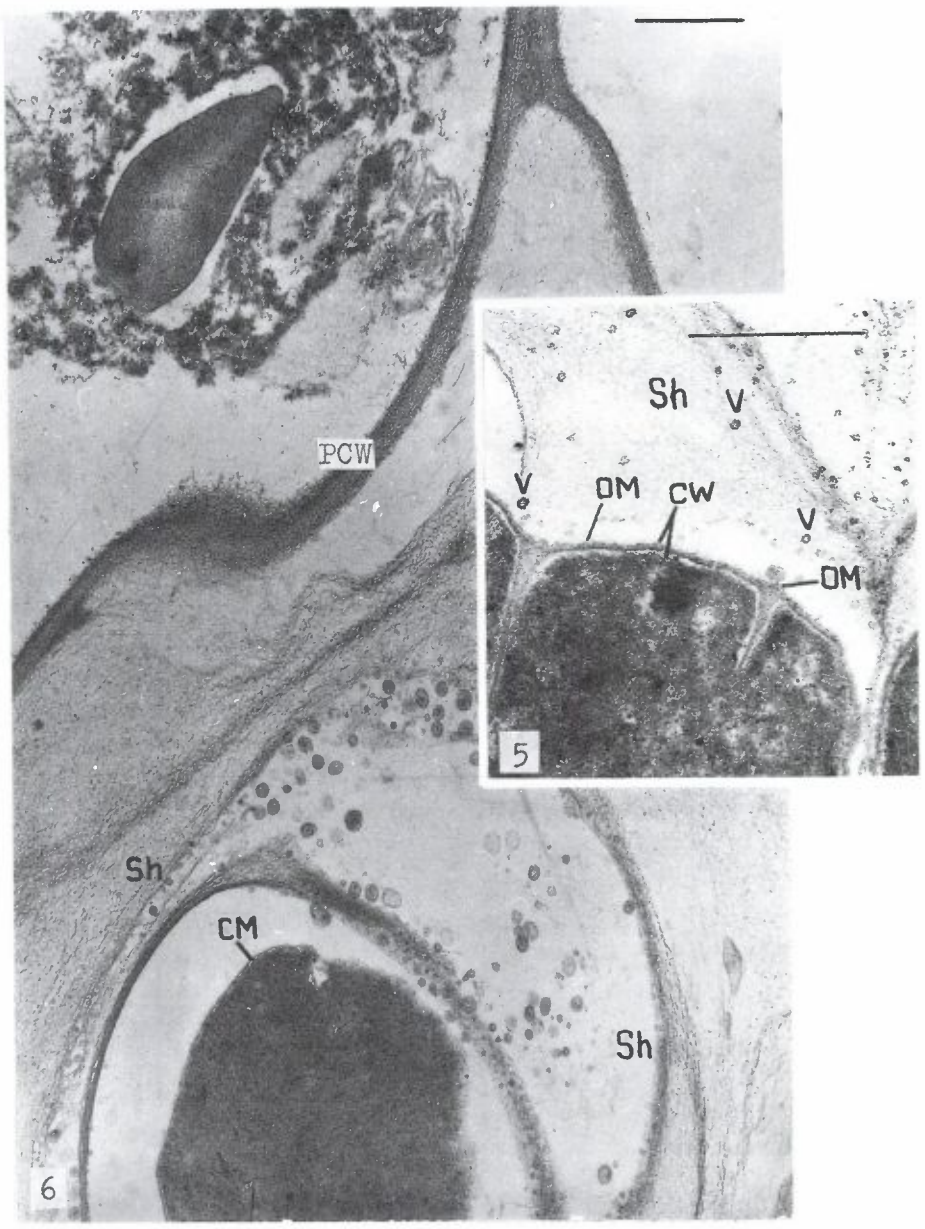


Figure 5. Part of *N. muscorum* filament in a harbor microcolony (var. 3N), the same alfalfa plant as in Fig. 3). Sh, sheath; CW, cyanobacterial cell wall; OM, outer membrane; V, vesicles (bar = 0.5 μm).

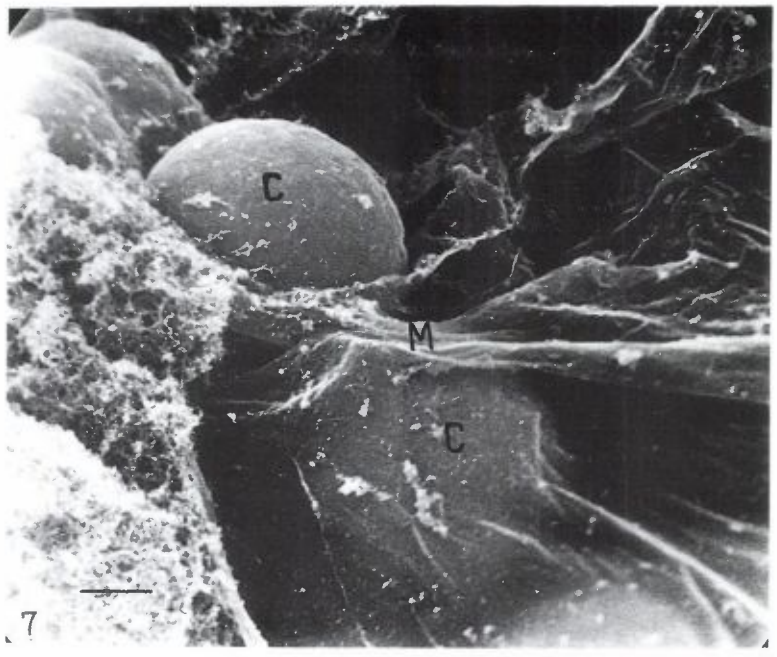
Figure 6. Part of *N. muscorum* protoplast in a harbor microcolony (var. 3N, the same alfalfa plant as in Fig. 3). Sh, sheath; V, vesicles; PCW, plant cell wall; CM, cytoplasmic membrane (bar = 0.5 μm).

the cyanobacterial sheath significantly expands and may fuse with the surface of plant cell walls (Fig. 4). The cell wall of cyanobacteria in the microcolony may remain a multilayer structure, as is typical of cyanobacteria (Fig. 5), but its rigidity is often reduced, and often the peptidoglycan layer is completely lost (Figs. 4,6). The outer membrane is being preserved or lost. In the latter case protoplasts form (Fig. 6). In some experiments this system (var. 3N) was formed in the absence of combined nitrogen. Under these conditions the heterocysts, additionally to vegetative cells and protoplasts, were revealed in the harbors of inoculated plants.

During formation of artificial syncyanoses not only do plant tissues reorganize, but an additional structure, homogeneous mantle-like layer, emerges which covers the cyanobacteria that settle on the surface of the plant partner. This was observed in the mixed suspension culture of ginseng and *C. fritschii* cells (Fig. 7). Independent partners never form such structures. The ruthenium red staining revealed that the mantle-like layer contains acid polysaccharides (data not shown).

The mantle-like layer emerges not only in the above suspension culture, but also on the surface of mixed alfalfa calli with *A. variabilis* (var. 1A) or *N. muscorum* (var. 1N) (Fig. 8). It also forms during settlement of these cyanobacteria on the surface of the alfalfa plant (var. 2A, 3N) (Fig. 3). Previously an analogous structure was revealed for the sites of *A. variabilis* ATCC 29413 location on the surface of tobacco callus and plants regenerated from a mixed culture (Gusev et al., 1986). The mantle-like layer favors closer integration of the partners. As the mantle-like layer emerges it isolates and thus protects the cyanobacteria from the unfavorable environment.

Additionally, associated growth of cyanobacteria and plants may result in intensified production of an amorphous mucilage; in particular, this occurs when the cyanobacterium *A. variabilis* grows on the root cap of alfalfa (var. 2A). The ruthenium red staining revealed that both partners contributed to the mucilage production (Figs. 9a,b). Figure 9a shows that the fibrilles which contain acid polysaccharides are extensions of the cyanobacterial outer lypopolysaccharide membrane. This suggests that the acid polysaccharide-containing mucilage is produced by the cyanobacteria. Mucilaginous fibrilles conglomerate with the formation of an electron-dense peripheral layer, surrounding the cyanobacterium cell (Figs. 9a,b). Thus, a thick mucilaginous sheath-like envelope emerges. It separates the cyanobacteria from the mucilage of the root cap in which they are immersed. Root cap mucilage also contains acid polysaccharides (Fig. 9b).



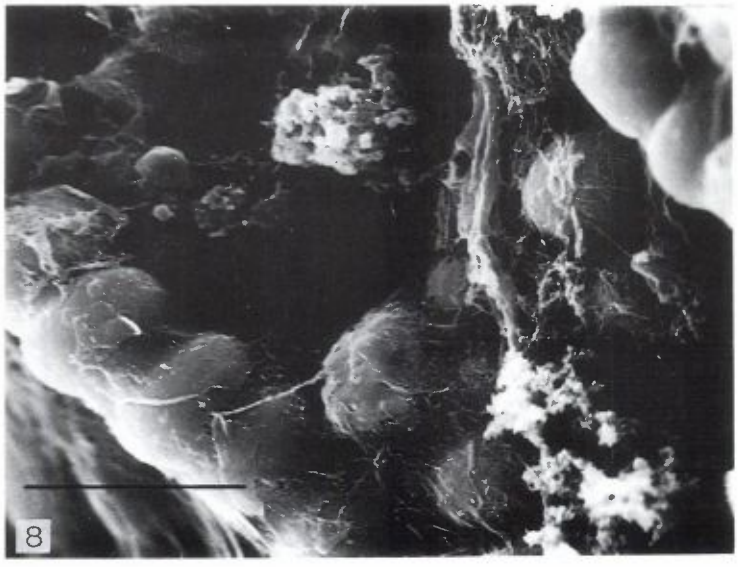


Figure 7. Mantle-like layer covering *C. fritschii* on the surface of *P. ginseng* aggregate. C, cyanobacterium; M, mantle-like layer (bar = 1 μm).

Figure 8. Mantle-lake layer covering *N. muscorum* on the surface of alfalfa callus (var. 1N); joint cultivation for 14 d (bar = 6 μm).

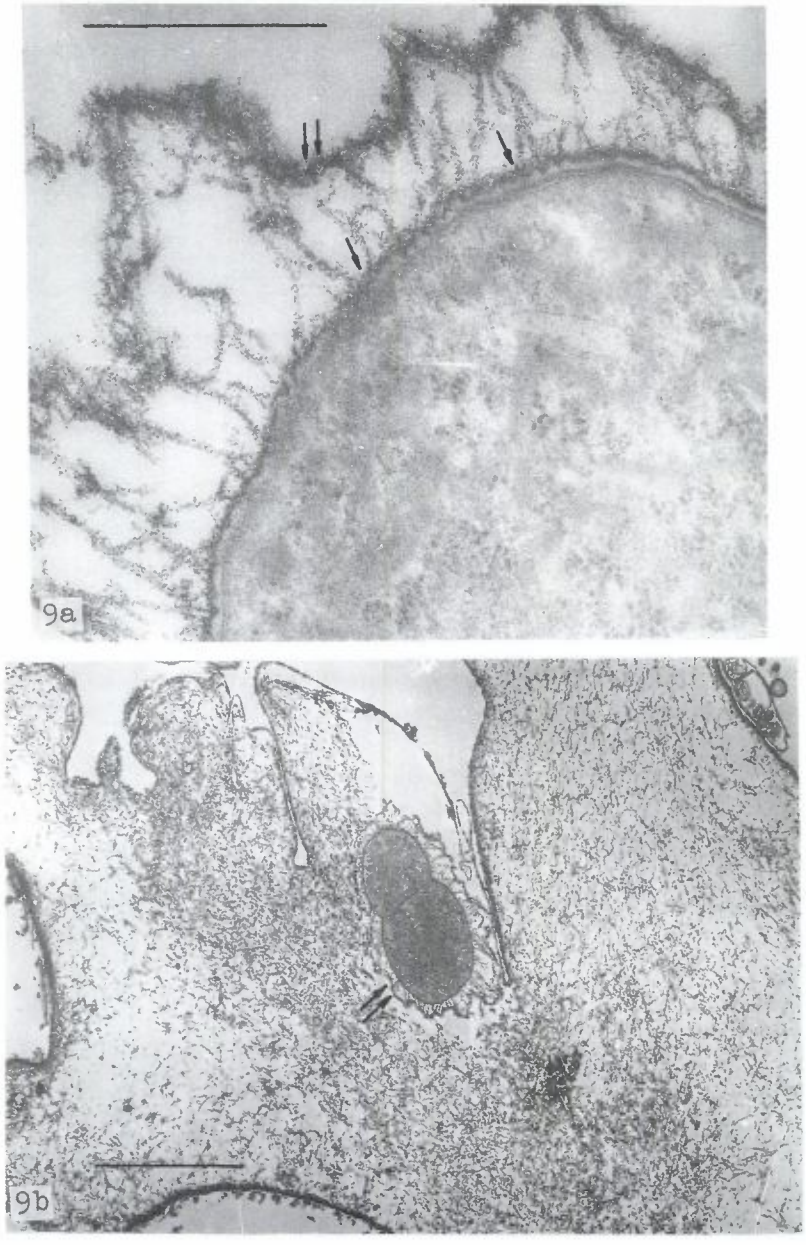


Figure 9. *A. variabilis* on the root cap of alfalfa (var. 2A), stained with ruthenium red; joint cultivation for 3 months: (a) polysaccharide fibrillas connected with the outer lypopolysaccharide membrane of cyanobacteria (single arrow) and forming electron-dense peripherical layer (double arrow) (bar = 0.5 μm); (b) *A. variabilis* cells immersed into the mucilage of alfalfa root cap; double arrow – electron-dense peripherical layer of cyanobacterial mucilage (bar = 1 μm).

Morphological modification of cyanobacteria in artificial syncyanoses

As the partners integrate morphologically in artificial syncyanoses, the heteromorphism of the cyanobacteria significantly increases, evidenced by the different morphological and ultrastructural organization of the cyanobacterial cells, cell clusters, colonies and subpopulations. Heteromorphic changes of cells in populations are apparently entailed by modifications of cyanobacteria surface structures, which will be described in this section. Mucilage and/or sheath production by cyanobacteria in the above artificial syncyanoses may be also classified with these changes.

Existence of cyanobacteria microcolony within the harbor (var. 3N) is accompanied by a significant change in the cell morphology, for the cell shape is irregular, the size varies, and the cells assemble into short filaments (Fig. 4). This pattern is identical with the morphology of cyanobionts in the *Nostoc* cavities of the bryophytes *Anthoceros* spp. and *B. pusilla* (Duckett et al., 1977). The cells with various degrees of cell wall reduction, including protoplasts, are characteristic of cyanobactria microcolonies in the harbor. What is important is that destructive changes of the protoplast cytoplasm, as a rule, were not observed (Fig. 6). Moreover, detachment of numerous membranous vesicles from the outer membrane and/or the cytoplasmic membrane suggests a possible functional activity of forms with reduced cell wall (Fig. 6). Numerous vesicles are also detached from the outer membrane of the multilayer cell wall of vegetative cells in a harbor (Fig. 5). The vesicles are filled with an electron-dense substance. The localization of vesicles of various sizes inside the sheath is probably attributable to construction of a growing sheath of cyanobacteria or to transfer of the substances between the plant and cyanobacteria cells. The possible transport function of the vesicles in lichens has been assumed in an earlier paper (Peveling, 1973).

Cells with a reduced wall and intact protoplasts have earlier been discovered by us in other artificial syncyanoses, in particular, in the intercellular spaces of the mixed callus of tobacco and *A. variabilis* CALU 458 that was transferred for a long time (Baulina et al., 1984). In *N. muscorum*, protoplasts form not only when cyanobacteria settle in mesophyll harbors (var. 3N), but also when they grow on the surface of the transferred alfalfa callus (var. 1N). The protoplast subpopulation was dominant in some *N. muscorum* colonies that were different by their habitus from the others and consisted of densely aggregated spherical cells and protoplast clusters. As a result, the colonies were grainy and their surface was warty. As mixed calli were further transferred, such colonies did not die but became mucilaginous, smooth and bright and included but few protoplasts characteristic for this association.

The morphological modifications of cyanobacteria in artificial syncyanoses are concurrent with reduction of the cell wall but also formation of specific surface structures in the course of cell differentiation, in particular, when heterocysts form. It is important that heterocysts were detected by us in syncyanoses of alfalfa with *N. muscorum* (var. 1N) or *A. variabilis* (var. 1A and 2A) even when cultivated on media which contained combined nitrogen, while heterocysts did not form in cyanobacteria monocultures growing under the same conditions.

Stimulation of heterocyst formation and nitrogenase activity of cyanobacteria associated with plants

Influence of plant partner on heterocyst formation by cyanobacteria and their nitrogenase activity was studied in alfalfa plants associated with *A. variabilis* (var. 4A) grown on nitrogen-free B5(5) medium. After associative growth under these conditions for 1 to 3 weeks, *A. variabilis* heterocyst frequency was 12–13% (Fig. 10). In *A. variabilis* monoculture, heterocyst frequency did not

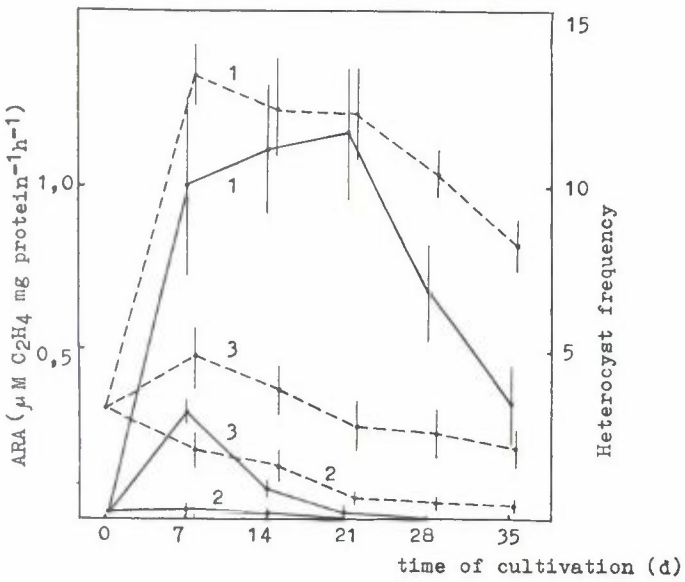


Figure 10. *A. variabilis* ARA (——) and heterocyst frequency (– – –) in association with alfalfa plants (var. 4A) during growth in nitrogen-free B5 (5) medium; 0 – the moment of inoculation. Before ARA measurement the roots infected with *A. variabilis* were cut from shoots and incubated with C_2H_2. 1 – associated roots (B5 (5) medium); 2 – *A. variabilis* monoculture (B5 (5) medium); 3 – *A. variabilis* monoculture (AA medium).

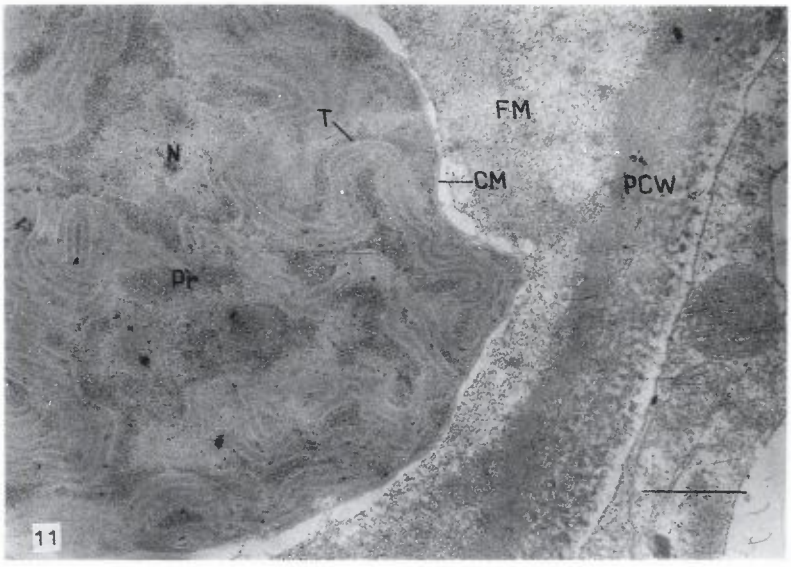


Figure 11. Protoplast of symbiotic *Nostoc* sp. in the thallus cavity of *B. pusilla*. CM, cytoplasmic membrane; T, thylakoid; Pr, polyribosomes; N, nucleoid; FM, fibrillar matrix; PCW, plant cell wall (bar = 0.5 μm).

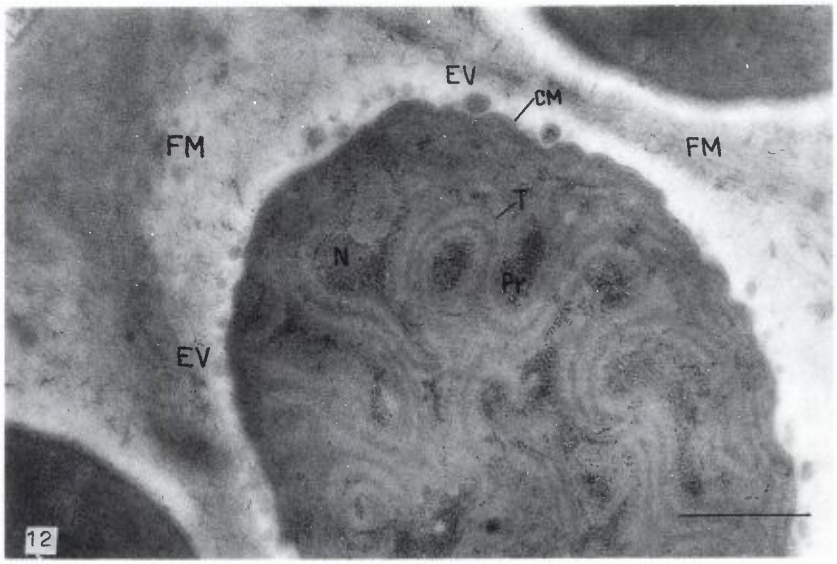


Figure 12. Protoplast of *Nostoc* sp. culture isolated from *B. pusilla*. The second transfer, cultivation for 2.5 months following isolation. CM, cytoplasmic membrane; T, thylakoid; Pr, polyribosomes; N, nucleoid; EV, exocytosis vesicles; FM, fibrillar matrix (bar = 0.5 μm).

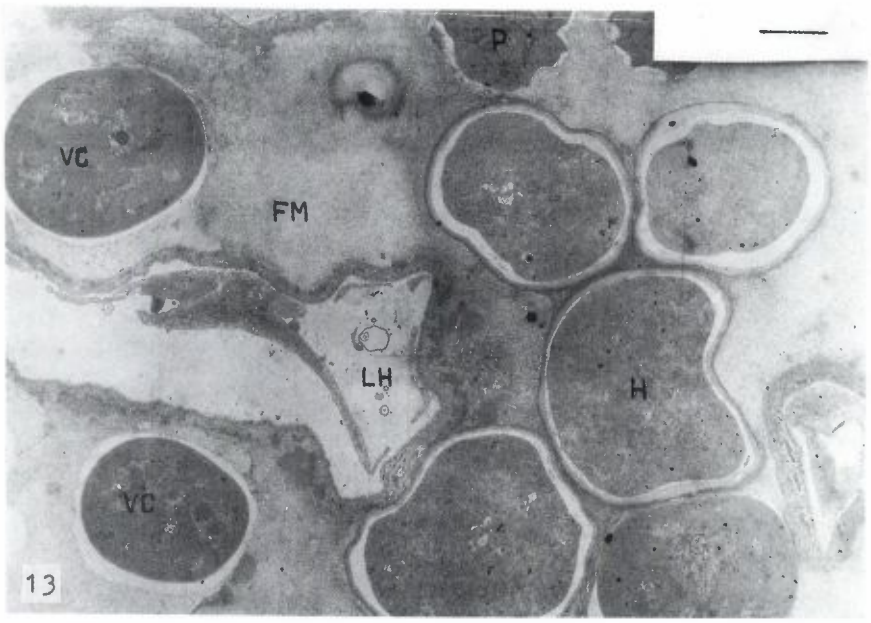


Figure 13. *Nostoc* sp. in *B. pusilla* thallus cavity. Vegetative cells (VC), heterocysts (H) and protoplasts (P) are immersed into a fibrillar matrix (FM). LH, liverwort hairs (bar = 1 μm).

exceed 2%. Acetylene reducing activity (ARA) of *A. variabilis* in association was thirty-forty times that in the monoculture under the same conditions. Moreover, heterocyst frequency and ARA level in associated cyanobacteria exceeded the same variables in the cyanobacteria monoculture in AA medium, optimal for its growth and nitrogen fixation (Fig. 10). These data are consistent with earlier results for associations of *A. variabilis* ATCC 29413 with alfalfa cultivated cells and tissues (Gusev and Korzhenevskaya, 1990), and also with tobacco tissues (Gusev et al., 1986). The results showed the increase of the same cyanobacteria variables in associations over those in cyanobacteria monocultures.

Morphological remodification of symbiotic cyanobacteria in artificial syncyanoses

Studies of the morphology and ultrastructure of symbiotic *Nostoc* sp. in liverwort *B. pusilla* detected protoplasts as well as vegetative cells and heterocysts. The protoplasts show, as a rule, no signs of degradation. Their characteristic features are an intact ultrastructure of the cytoplasmic membrane, thylakoids, polyribosomes, nucleoid and cytoplasmic matrix (Fig. 11). Protoplasts of a

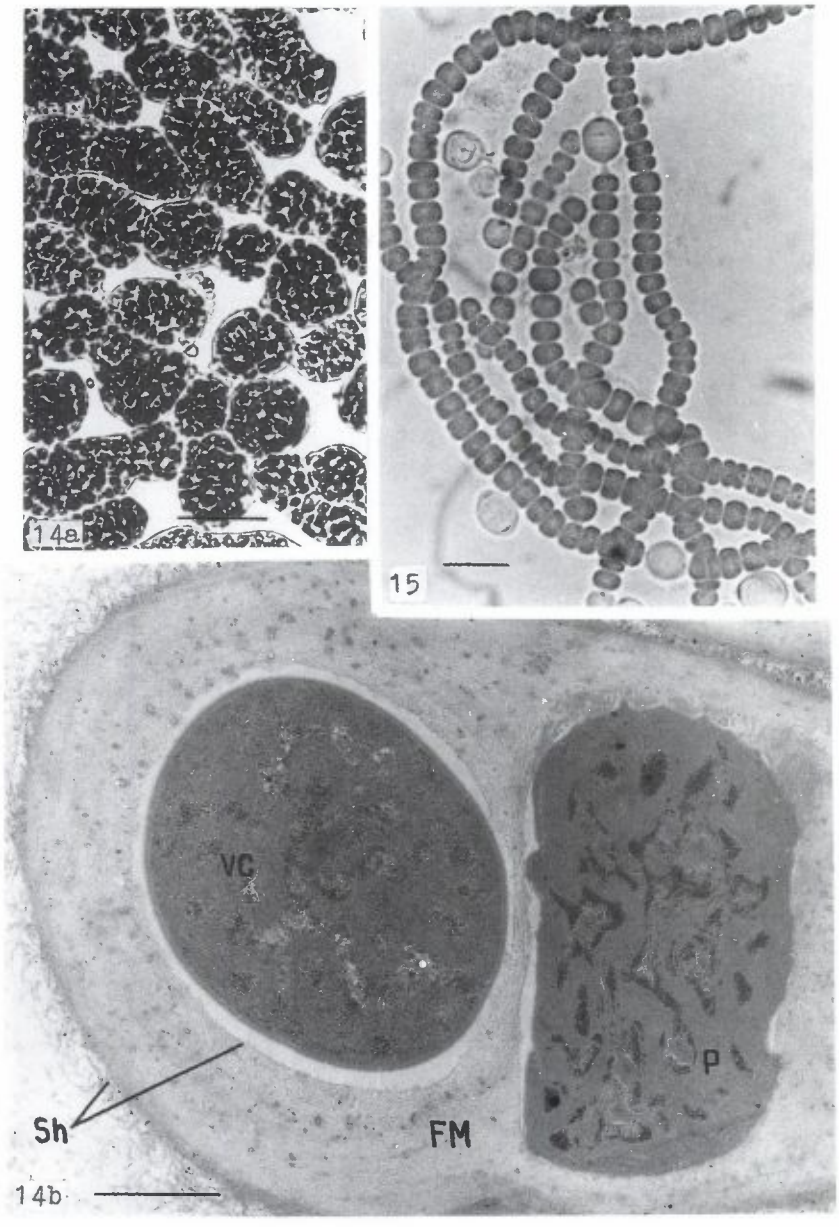


Figure 14. Morphology of *Nostoc* sp. isolated from *B. pusilla*. The second transfer, cultivation for 2.5 months following isolation: (a) light microscopic micrograph of ensheathed clusters (bar = 10 μm); (b) thin section micrograph. VC, vegetative cell; P, protoplast; FM, fibrillar matrix; Sh, sheath (bar = 1 μm).

Figure 15. Light microscopic micrograph of *Nostoc* sp. isolated from *B. pusilla*. Transferred for 14 months following isolation. H, heterocyst (bar = 5 μm).

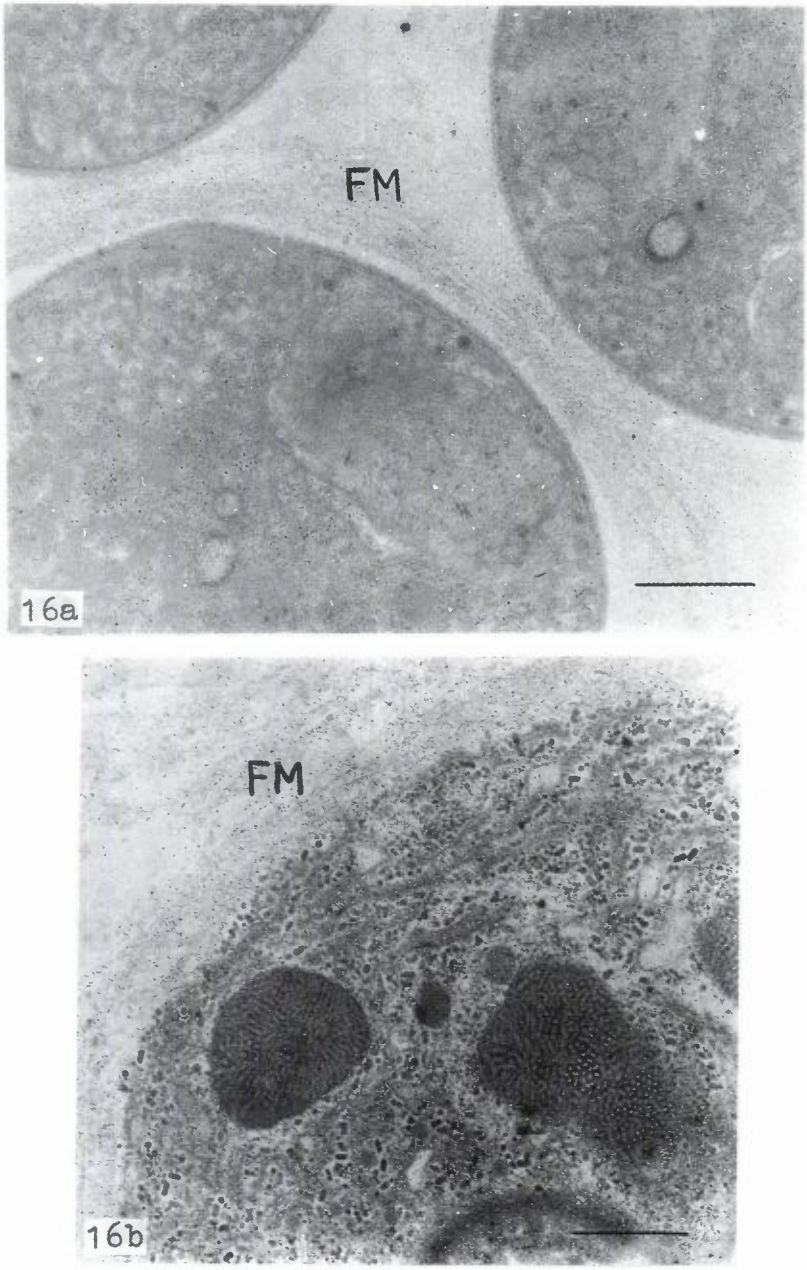


Figure 16. *Nostoc* sp., isolated from *B. pusilla*, in mixed culture with rice callus, joint cultivation for 17 d: (a) single vegetative cells. FM, fibrillar matrix (bar = 0.5 μm); (b) "plasmatic forms" without envelope. FM, fibrillar matrix (bar = 0.5 μm).

similar structure are often found in the first two transfers of isolated *Nostoc* sp. (Fig. 12). Besides, active exocytosis proceeds in them. This suggests that the protoplasts are alive and active.

Vegetative cells, heterocysts and protoplasts in liverwort are immersed into a dense fibrillar matrix, an "intercellular matrix" (Figs. 11,13). They are localized in the intercellular matrix as singular units or small groups. In the first two transfers of isolated *Nostoc* sp. vegetative cells, heterocysts and protoplasts, immersed into fibrillar intercellular matrix, form compact ensheathed clusters (Figs. 14a,b). The peripheral layer of a cluster sheath is formed by a dense fibrillar matrix. Subsequent subcultivation of cyanobacteria eliminated protoplasts and the matrix. The ensheathed clusters were replaced by trichomes (Fig. 15), that also has been observed in culture of *Nostoc* sp. isolated from the lichen *Nephroma laevigatum* (Kardish et al., 1989).

Transferred *Nostoc* sp. isolated from *B. pusilla* was used in inoculation of plant tissues. When they grew in contact with a callus rice tissue, cyanobacteria changed morphologically and the ultrastructure of cell envelope (cytoplasmic membrane, peptidoglycan layer, outer membrane, sheath) reorganized. On numerous occasions the cell envelope underwent complete degradation. The trichomes disappeared. Fibrillar matrix emerged around single cells and "plasmatic forms" without envelope (Figs. 16a,b). Consequently, morphology of *Nostoc* sp. in artificial syncyanoses with rice tissue resulted in an organization similar to that in liverwort thallus.

Acquired obligate dependence of the cyanobiont on callus culture

The artificial syncyanoses in our experiments were highly stable during the long period of mixed cultivation, for instance they existed as many as 23 transfers in a mixed callus culture (Gusev et al., 1986). Usually the partners can be cultivated separately under conditions optimal for them. In a single case (var. 1N) *N. muscorum* could not be isolated into pure culture following 10 months of existence in associated alfalfa callus (about 10 transfers) and maintained this feature for 18 months.

4. Discussion

The above artificial associations that were created from plant units and cyanobacteria possess the following new functional and structural features:

1. formation of a specific anatomical organization;
2. mucilage mantle formation;
3. modification of the cyanobiont cell wall;

4. increase of the cyanobiont heterocyst frequency;
5. increase of cyanobiont ARA level;
6. morphological remodification of symbiotic cyanobacteria;
7. obligate dependence of the cyanobiont on the host.

The analysis of earlier experimental data reveals, in addition to the above, other new functional features of artificial associations.

8. Growth of associations under deficiency or lack of organic C or combined N. The artificial associations in which the cyanobacteria acted as a phototrophic partner were subjected to light in modified MS or B5 media with the initial sucrose concentration reduced by a factor of 16 (Butenko et al., 1982; Gusev et al., 1982). Under these conditions in monocultures cyanobacteria and plant cells grew slowly if at all. In mixed suspension cultures in organic carbon-deficient media the division and growth of plant cells were observed to be stimulated and sufficiently exceeded those in corresponding monocultures. The use of ^{14}C-labelled bicarbonate showed that photosynthetic products excreted into the medium by *C. fritschii* supported the plant cell growth (Korzhenevskaya et al., 1984).

Artificial associations with cyanobacteria as a nitrogen-fixing component were obtained in media favorable for growth of plant material on nitrogen-deficient or nitrogen-free media. Plant cells were observed to divide, and growth of suspension and callus cultures occurred in the presence of nitrogen-fixing cyanobacteria in nitrogen-free media (Gorelova et al., 1984; Gusev et al., 1986). Plants infected with cyanobacteria were capable of sustained growth with lack of combined nitrogen on an agar medium for nine months and in a sand culture for three months of observation (Gusev and Korzhenevskaya, 1990). Control (noninfected) plants died within a month under the same conditions.

9. The synthesis of species-specific substances by plant cells in association with cyanobacteria. Cyanobacteria in mixed cultures make plant cells change the amount of species-specific products that they synthesize (Gusev and Korzhenevskaya, 1990). Cultivated *Solanum laciniatum* cells are producers of nitrogen containing substances, i.e. steroid alkaloids. In a nitrogen-free medium which does not support the growth of the culture, alkaloids do not accumulate. On the other hand, in association with nitrogen-fixing *C. fritschii*, plant cells not only preserve their ability to divide but also synthesize alkaloids. The maximal alkaloid content in a mixed culture was 89% higher than that in *S. laciniatum* monoculture under similar conditions (Gorelova et al., 1984).

These nine features of artificial associations are newly acquired ones. Acquisition of new properties is also characteristic of natural symbioses (Margulis, 1981). Most of the features of artificial associations are similar to those of natural syncyanoses.

One of them is their ability to grow in nitrogen deficient/free media or under deficiency of organic carbon. In natural syncyanoses cyanobacteria also act as photosynthesizing and/or nitrogen-fixing components (Reisser, 1984; Rai, 1990b). We succeeded in the creation of artificial associations with the cyanobacterial partner performing various metabolic functions similar to those in natural syncyanoses. The similarities with natural symbioses were displayed also in similarities of conditions for formation of artificial associations. *In vitro* creation of an artificial association, as well as formation of a symbiosis in nature (Margulis, 1981; Ahmadjian and Jacobs, 1983), proceeded under conditions unfavorable for growth of one of the partners or both of them (Butenko et al., 1982; Gusev et al., 1982). New properties discussed above provided advantages for the partners in association over nonassociated organisms.

As in natural syncyanoses with a photosynthetic partner (Rai, 1990a), in our experiments in artificial associations the heterocyst frequency and ARA increased significantly in the cyanobiont over those in free-living cyanobacteria.

Similarity with natural symbioses is also seen in mucilage formation in cyanobacteria settlement sites which is usually observed in natural rhizocenoses where both plants and bacteria contribute to the process (e.g. Greaves and Darbyshire, 1972). In natural symbioses of cyanobacteria and plants mucilage also forms in large amounts (Obukowicz et al., 1981).

Cyanobacteria with reduced cell walls also exist in natural symbioses with cycad plants (Grilli Caiola, 1980; Grobbelaor et al., 1988). They may be the natural analog of cyanobacteria cells with a modified cell surface found in artificial associations.

Symbiotic cyanobacteria are known to be isolated from the plant and be cultivated as isolates (Stewart et al., 1983). One exception is the assumed obligate dependence on the host in the case of a dominant *Anabaena azollae* symbiont in natural symbiosis with *Azolla* fern (Meeks et al., 1988). In the only case of artificial association cyanobacteria acquired the obligate dependence on the plant partner.

In artificial syncyanoses cyanobacteria occupy the natural spaces of plants, and their location in intercellular spaces of cell aggregates in suspension and callus cultures, on the surface and in intercellular spaces, stomatal pores, vascular system of plant leaf, stem and root tissues has been previously described (Gusev et al., 1984; Gusev et al., 1986; Gusev and Korzhenevskaya, 1990). In this paper we have described the creation of a new anatomical organization of

artificial syncyanoses for settlement of cyanobacteria. There are two obvious trends in structural organization of artificial syncyanoses. The first is the close integration of the partners, and the second is the symbiont compartmentation in local zones of the plant partner. In natural syncyanoses the cyanobiont is also separated by certain structures from the host; their formation is, however, genetically determined (Stewart et al., 1983). The "harbors" for cyanobacteria in experimental systems may be viewed as imitation of early stages in creation of structures for compartmentation of the microsymbiont.

These similarities of artificial and natural syncyanoses suggest various applications of the method of artificial associations to modeling and analysis of natural symbioses.

Firstly, analogies of the cyanobiont functioning in natural and artificial associations as a photosynthesizing or a nitrogen-fixing partner of the plant and modification of cyanobacteria under the impact of the plant partner, may be useful in modeling and analysis of symbiotic interactions on the cell and tissue levels. These systems may provide insights into factors of heterocyst differentiation and induction of high ARA level in cyanobacteria (Rozen et al., 1986), into the origin and functional roles of mucilage in cyanobiont settlement areas (Bonnett, 1990), into factors affecting the changes in the cell wall, and into metabolic and genetic interaction of the partners.

Secondly, symbiotic cyanobacteria which lost their "symbiotic" features when isolated, remodified their morphological properties, once associated with a nonsymbiotrophic plant. This is seen, in particular, as similarities of the structural organization of symbiotic cyanobacteria in natural symbiosis and that of a natural isolate in artificial association with a nonsymbiotrophic plant. In this light another application of the method is preservation of the symbiotic status *in vitro* of a symbiotroph isolated from a natural symbiosis. A symbiotic organism is quite different when isolated and in a natural symbiosis by numerous criteria (Smith and Douglas, 1987). Artificial association with a foreign host may prove the only way to maintain and, possibly, accumulate the symbiont *in vitro*. For these purposes Meeks et al. (1988) succeeded in reconstructing symbiosis of the hornwort *Anthoceros punctatus* with an isolate from the fern *Azolla caroliniana*. The minor component of an *A. azollae* population believed to be selected and accumulated when the isolate is infected and cultivated in hornwort whereas the dominant form of the cyanobacteria cannot be cultivated outside the fern.

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