Microbiology, Vol. 69, No. 4, 2000, pp. 471–479. Translated from Mikrobiologiya, Vol. 69, No 4, 2000, pp. 565–573.

EXPERIMENTAL ARTICLES

Spatial Integration of the Partners and Heteromorphism of the Cyanobacterium *Nostoc muscorum* CALU 304 in a Mixed Culture with the *Rauwolfia* Tissue

O. A. Gorelova

Department of Cellular Physiology and Immunology, Faculty of Biology, Moscow State University, Vorob'evy gory, Moscow, 119899 Russia
e-mail: creod@orc.ru

Received October 20, 1999

Abstract—A comparative morphological study was conducted of *Nostoc muscorum* CALU 304 grown either as a pure culture on standard media or as a mixed culture with *Rauwolfia* callus tissue on a medium for plant tissue cultivation. The interaction of the cyanobacterial and plant partners results in their spatial integration into aggregates of specific anatomy, which arise periodically during the mixed culture growth. The morphology of the cyanobacterial cells varies depending on their localization in the mixed aggregate. The degree of cyanobacterial heteromorphism increases with the time of growth of the association. Evidence of the plant origin of the factors inducing heteromorphic changes in *N. muscorum* was obtained, as well as evidence indicating that these factors can rapidly diffuse in agarized medium. A conclusion is inferred that the heteromorphic cells correspond to bacterial forms that appear during unbalanced growth as an adaptation to altered environmental conditions.

Key words: cyanobacteria, plant tissues, symbiosis, artificial associations, *Nostoc*, heteromorphism, modifications.

Nostoc spp. are the most widespread microsymbionts contributing to the plant and fungal syncyanoses [1, 2]. The high symbiotic potential of cyanobacteria of this genus was also revealed in the experiments on the reconstitution of natural symbiotic associations and the creation of artificial ones between various cyanobacteria and plants or their cells and tissues cultivated in vitro [1, 3–11]. Unlike representatives of the other ten genera studied, *Nostoc* sp. of nonsymbiotic origin can infect the intrathallus cavities of the moss *Anthoceros punctatus* [6] and the stem glands of the angiosperm *Gunnera manicata* [7], forming reconstructed syncyanoses under laboratory conditions. Stable artificial associations were reported to be formed by *Nostoc* sp. strains 2S3B and 2S9B with wheat roots [8], by *Nostoc muscorum* VKM 16 with cells, tissues, and plants of aefalfa [9, 10], and by *Nostoc muscorum* CALU 304 with nightshade tissues [11].

De novo formation of syncyanoses, both natural and artificial, is accompanied by metabolic, morphological, and ultrastructural modifications of cyanobacteria due to their spatial and metabolic integration into the combined symbiotic system [1–12].

The mixed culture of the callus tissue of *Rauwolfia* and *Nostoc muscorum* CALU 304 is a model association used to study changes in cyanobacteria caused by their interactions with the plant. We have previously described some of the adaptive modifications of the organisms in this association [13–15]. The effect of the plant partner is manifested in the stimulation of cyanobacterial growth under unfavorable conditions and in heterocyst differentiation and induction of nitrogenase activity during growth on nitrogen-containing medium. There were also changes in the dynamics of reserve polymer accumulation in the vegetative cells.

This paper presents the results of a morphological study of the process of spatial integration of the partners in a mixed culture of *Rauwolfia* callus tissue and *Nostoc muscorum* CALU 304.

MATERIALS AND METHODS

An axenic culture of the free-living nitrogen-fixing cyanobacterium *Nostoc muscorum* Agardh., strain CALU 304, was grown as described previously [11] using the nitrogen-containing BG-11 medium [16] and the nitrogen-free Allen–Arnon medium (AA medium) [17] (the media and conditions used to grow a pure culture of cyanobacteria are denoted as standard). A mixed culture of a callus tissue of a nonsymbiotrophic plant *Rauwolfia serpentina* Benth., strain K-27, and *N. muscorum* CALU 304 was obtained and cultivated as described previously [14]; in that paper and in this, the cyanobacterial culture grown under the same conditions as the mixed culture is referred to as a monoculture.

The following variants of cultivation were used to reveal changes in the morphological characteristics of

0026-2617/00/6904-0471$25.00

Differentiated and heteromorphic cells in the *N. muscorum* CALU 304 populations under various growth conditions

Variant		Cell types[1]					Amorphous mass
culture	incubation time, days	Hc	Ak	FDCW	Gi	Mc	
Pure							
On BG-11	22	–	–	+	–	+	+
On AA	27	+	+	+	–	+/–	+
Monoculture							
	16	–	+	+	–	+	+/–
	58	–	+	–	–	–	–
Mixed[2]							
S	16	–	+	+	–	+	–
A	41	+	+	+	+/–	+	+
B	41	+	–	–	–	+/–	–
C	41*	+	–	–	–	+/–	–
C	41**	+	+	+	–	+/–	–
A	58	+	+	+	+	+	+
B	58**	+	+	+	+	+	+
Mixed (no contact with plant tissue):							
	16	–	+	+	–	+	–
	70	+	+	+	+	+	+

[1] Heterocysts (Hc), akinetes (Ak), forms with defective cell wall (FDCW), giant cells (Gi), and minicells (Mc).
[2] Data on the cells from the following zones are presented: S, the primary microcolony on the surface; A, B, and C, zones A, B, and C of the mixed aggregate, see Fig. 2.
* and **, cyanobacteria from young and mature surface colonies of the mixed aggregate, respectively.

N. muscorum: (1) a pure culture on BG-11 and AA media; (2) a monoculture on the medium for *Rauwolfia* cultivation (hereafter referred to as P medium); (3) a mixed culture, obtained by applying cyanobacteria on the callus surface followed by incubation on P medium; (4) a mixed culture on the P medium surface without contact of cyanobacteria with the callus tissue (in this case, the partners were spatially separated and their interaction occurred via metabolite exchange by diffusion through the agarized medium). Note that the P medium suitable for the growth and biosynthetic activity of the plant tissue is unfavorable for cyanobacteria, because its nutrients are excessive and unbalanced for cyanobacteria. In particular, sodium is absent from this medium; it contains products of casein hydrolysis (amino acids and peptides), and the content of mineral nitrogen is five times higher than that in the nitrogen-containing BG-11 medium, whereas the content of sucrose is 20–25 times higher than the concentration required for photoheterotrophic growth of cyanobacteria isolated from natural symbioses [2].

Specimens prepared as described previously [10] were examined using light microscopy and transmission and scanning electron microscopy to reveal differentiated cells and describe the morphology of cyanobacterial populations.

In total, 12 experiments were conducted (three series); each experiment was performed in three to nine replicates.

RESULTS AND DISCUSSION

Morphological and ultrastructural features of the strain *N. muscorum* CALU 304 grown under standard conditions corresponded to those described in our previous papers [11, 14, 15] and are typical of the representatives of the genus *Nostoc*. In addition to unchanged vegetative and differentiated cells, some heteromorphic cells were found in a stationary-phase cyanobacterial population, namely, forms with defective cell walls (FDCW), from cells with reduced rigidity of the peptidoglycan layer to protoplasts, and minicells (Mc) that were 0.7–1.5 μm in diameter, spheric in shape, and had intact cell walls (table).

After incubation for two weeks on P medium, the morphological distinctions between cyanobacteria in monoculture and mixed culture were insignificant (table). Cyanobacteria appeared as chains, single vegetative cells, akinetes, FDCW, and often minicells (Fig. 1). As a rule, minicells were connected with trichomes in the sites of cell division; more seldom, they were free. Cells of all types had well developed mucous sheaths.

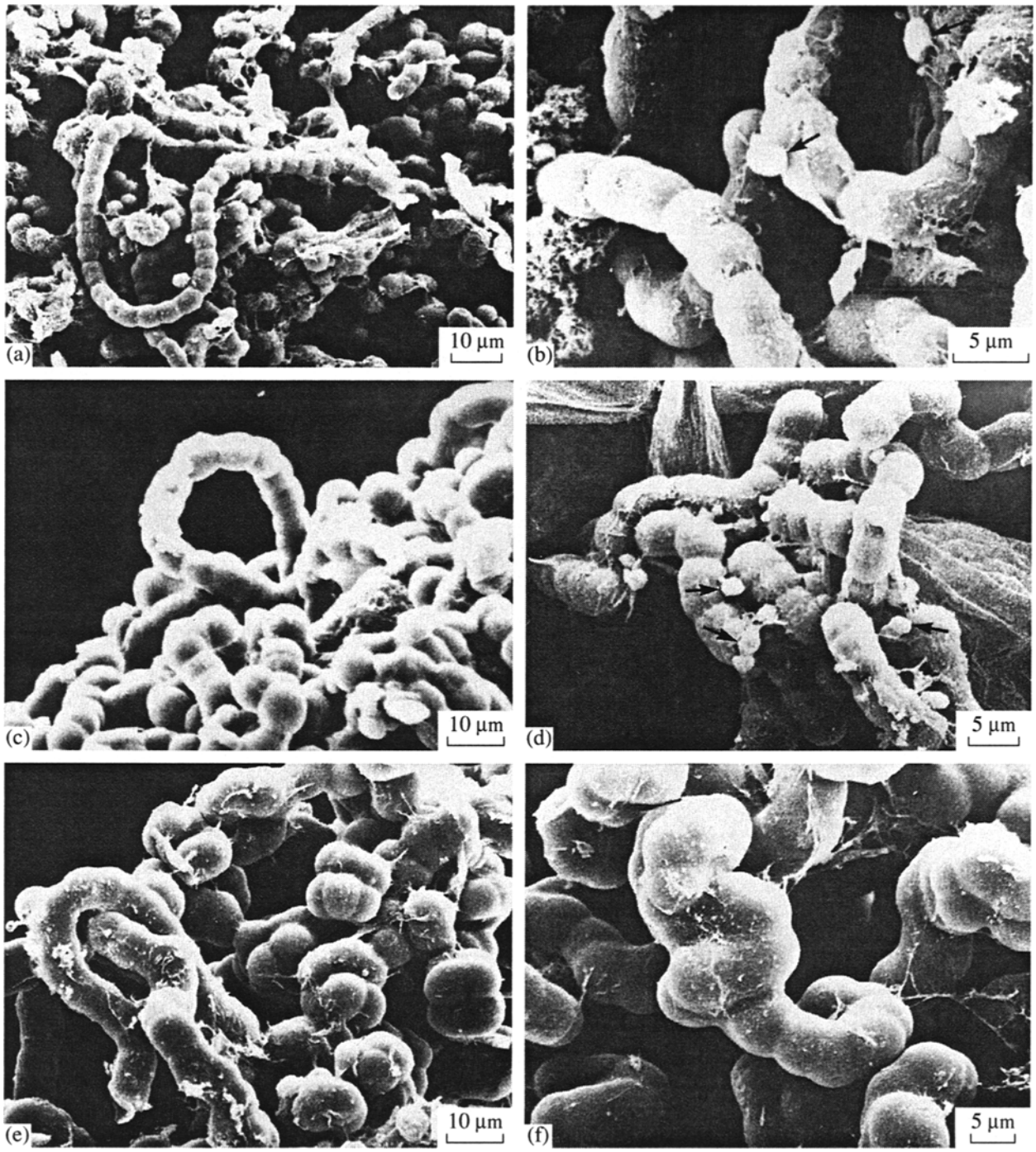

Fig. 1. Morphology of *N. muscorum* CALU 304 after 16 days of incubation on the P medium in (a and b) the monoculture and (c–f) the mixed culture. (c, d) Cells in the primary surface microcolony on the rauwolfia callus; (e, f) cells that do not contact the callus tissue. Minicells are indicated with arrows.

Trichomes were often curved and twisted and appeared as clusters, tetrads, or multiseriate and sometimes branching formations (Fig. 1e and 1f). We previously reported the occurrence in this culture of not only false but also true branching, resulting from cell division in more than one plane [15].

During subsequent growth, the cyanobacterial morphology depended on the cultivation variant.

In a monoculture, where no increase in biomass occurred for 1.5–2 months [14] and cell division was rare, the heteromorphic cells disappeared (table).

In the mixed culture, active growth of *N. muscorum* was observed. The cyanobacteria, applied initially on the callus surface as a suspension, formed primary microcolonies; then, their hormogonia entered the intercellular spaces and penetrated the plant tissue.

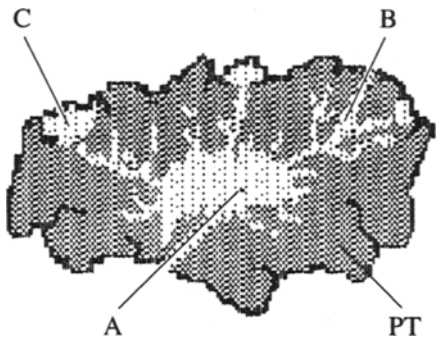

Fig. 2. Scheme of the mixed aggregate. A, B, and C are localization zones of *N. muscorum* CALU 304 cells; PT is the plant tissue.

However, the distribution of cyanobacteria within the callus tissue was not disperse. The spatial integration of partners was completed in three to four weeks of the associated growth by the formation of mixed aggregates with a specific morphological structure [13, 14].

In the mixed aggregate, three zones of cyanobacteria were easily distinguishable by the end of the sixth week (Fig. 2). Within the aggregates, *N. muscorum* forms a central microcolony (zone A), which is localized in widened intercellular spaces (Fig. 3a) or sometimes in dead *Rauwolfia* cells (Fig. 3b). In this zone, cyanobacteria are arranged in short chains, consisting of 3–7 large rounded cells or are represented by single heteromorphic cells and hormogonia. Some *N. muscorum* cells are located near the cellulose frameworks of surrounding plant cells, whereas other cells are free. On the periphery, the central cyanobacterial microcolony is surrounded by numerous interweaved hormogonia and vegetative trichomes consisting of 15–40 cylindric cells (Figs. 3c, 3d). We denoted this zone of cyanobacterial growth in the mixed aggregate as zone B (Fig. 2). Hormogonia and trichomes penetrate the narrow intercellular spaces between tightly packed *Rauwolfia* cells, which are smaller than the cells of the surrounding callus tissue. Neither akinetes nor heteromorphic cells were observed in this zone (table). Minicells were extremely rare. Neither was heteromorphism characteristic of cyanobacteria localized in zone C, i.e., on the aggregate surface (Figs. 3e, 3f). Here, mostly chains of round cells of medium size occurred. The small portion of hormogonia occurring among these cells decreased with time. A few akinetes, FDCW, and minicells were also revealed (table). Beginning with the fifth or sixth week of the associated growth, heterocysts exhibiting nitrogenase activity [13, 14] were encountered among cyanobacteria in all zones of their localization (Fig. 3).

Before the seventh week of incubation, *N. muscorum* vegetative cells and heteromorphic forms within the mixed aggregates had poorly developed sheaths or completely lacked them. In the aggregates, we revealed neither slime accumulation nor the formation of the extracellular matrix found previously in the specialized symbiotic thallus cavities of the *Blasia pusilla* moss [10, 12] or in cavities of the leaves of alfalfa grown in association with *N. muscorum* VKM 16 [10, 20].

The mixed aggregates developed with time; both their aging and de novo formation were observed during the mixed culture growth. Since the proliferative activity of plant cells surrounding the surface microcolonies of *N. muscorum* increased periodically, the microcolonies found themselves in zone A. Cyanobacteria localized in the depth of the callus (zones A and B of the previous aggregate) could no longer be revealed because, most probably, they were eliminated or transferred (in the form of hormogonia) to the new aggregate. This was followed by decelerated growth of the plant tissue, whereas cyanobacteria intergrew to the surface of the new aggregate. Several other artificial associations between cells and tissues of higher plants and cyanobacteria were previously found to display the alternating activation of either one or the other partner; however, no aggregates having the structure described in this work were formed [1, 18].

The cyanobacterial morphology in an aggregate formed de novo is similar to that described above (Figs. 4a and 4c). However, the proportion of heteromorphic cells and their diversity increased (table). The population heterogeneity was extremely pronounced in zone A (Fig. 4c). Even the vegetative cells differed significantly here. Chains of cells from 2–3 to 8.5–9 μm and even to 12–13 μm in diameter were simultaneously encountered in the same preparation. Giant forms up to 24.5 μm in diameter were also found. Most cells were round in shape, although disk-shaped cells of a lesser size than that of the round cells were often present (Fig. 4c). The disk-shaped cells were 1.5–2 μm long and 3–4 μm wide. We determined the size of stationary-phase vegetative cells of the *N. muscorum* CALU 304 grown under standard conditions to be 5.58 ± 0.22 μm (length) by 2.9 ± 0.25 μm (width) [14]. The changes in size and form of the vegetative cells described in the present work probably result from an imbalance between the growth and division processes. As a rule, trichomes consist of equal-sized cells. We suggest that at a reduced frequency of cell divisions, cell growth is accompanied by cell enlargement; whereas at a high frequency of cell division, the chains of small cells are formed. The sheath morphology was also diverse. They could be thick, one- or multilayered, could have local zones of lysis and large breaks, or could be completely absent. The extracellular slime was only detected in small amounts (Figs. 4a–4c).

Thus, the morphology and ultrastructural organization of *N. muscorum* was determined not only by the duration of the associated growth but also by the bacterial localization in an aggregate and by the life span of the given microcolony. The relative age of cyanobacteria in the central microcolony (zone A) exceeds the age

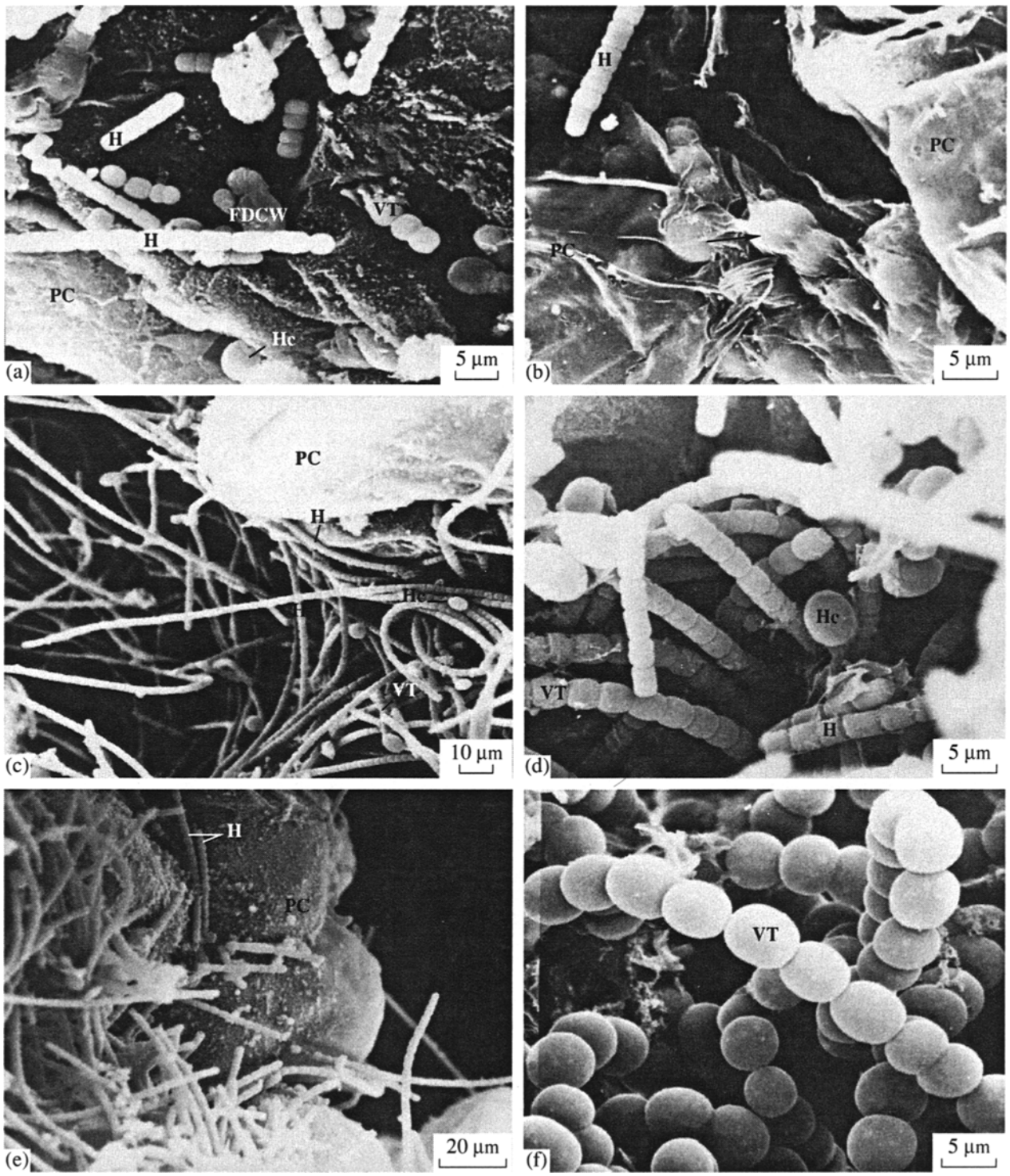

Fig. 3. Morphology of *N. muscorum* CALU 304 in the mixed aggregate (41 days): (a, b) in zone A in widened intercellular spaces and in a dead rauwolfia cell (indicated with an arrow); (c, d) in zone B and (e, f) zone C, in a young and mature microcolony, respectively. PC, a plant cell; H, a hormogonium; VT, a vegetative trichome; Hc, a heterocyst; FDCW, a form with a defective cell wall.

of cyanobacteria in the surface microcolonies (zone B). In young microcolonies, the number and diversity of heteromorphic cells are limited; with an increase in the age of a microcolony, the populational heteromorphism becomes more pronounced (Figs. 1, 3, and 4).

When growing in mixed aggregates, *N. muscorum* cells occur under variable physical conditions (e.g., variable illumination); the effect of the plant tissue on the bacteria is, probably, also variable. When the bacterial suspension is placed as a drop on the medium surface, direct contact between the bacteria and callus is

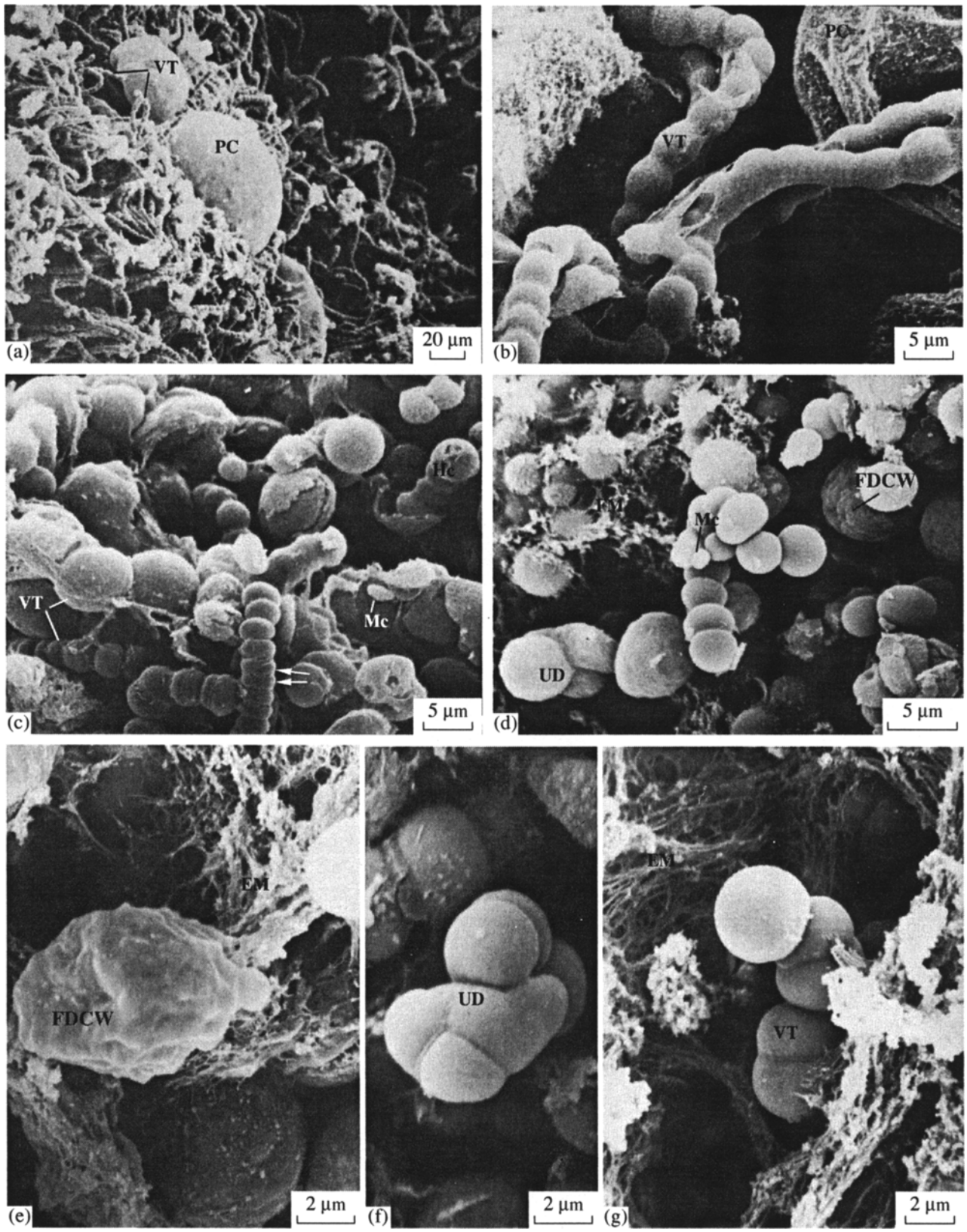

Fig. 4. Morphology of *N. muscorum* CALU 304 in the later incubation time of the mixed culture: (a–c) in zones C, B, and A of the mixed aggregate, respectively (58 days); (d–g) during growth without contact with the plant tissue (70 days). PC, a plant cell; H, a hormogonium; VT, a vegetative trichome; Hc, a heterocyst; FDCW, a form with defective cell wall; Mc, a minicell; UD, a cell with uncompleted division; EM, extracellular matrix. Disk-shaped cells are indicated with arrows.

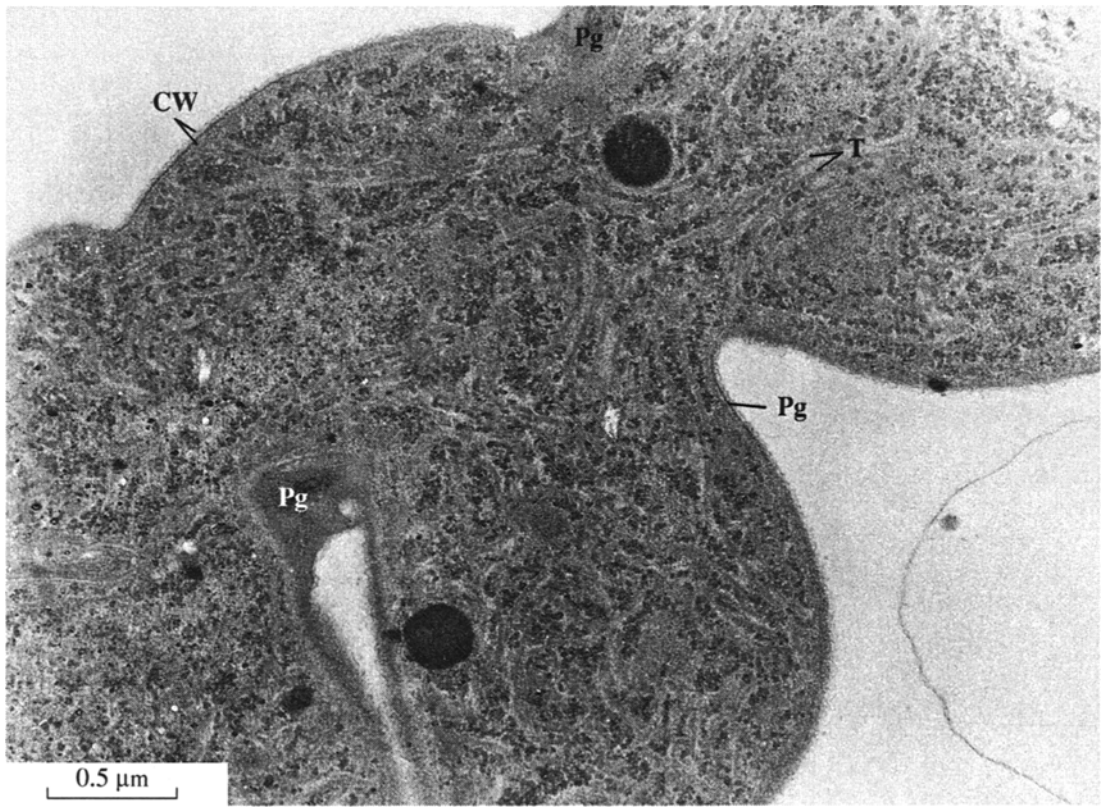

Fig. 5. Fragment of an irregular *N. muscorum* CALU 304 cell that has undergone several uncompleted divisions. CW, cell wall; Pg, peptidoglycan, T, thylakoids.

impossible. Under these conditions, cyanobacteria occur under more stable growth conditions; the influence of the *Rauwolfia* callus gradually increases with time, because the plant growth is accompanied by utilization of nutrients and diffusion of metabolites (including biologically active ones) secreted by viable plant cells or released from dead plant cells [13].

We previously demonstrated that, under these growth conditions, the cyanobacterial system regulating the nitrogen metabolism undergoes changes analogous to those occurring in *N. muscorum* CALU 304 cells in the mixed aggregates [14]. *N. muscorum* grew in the form of isolated convex round mucous colonies, which enlarged in size two- to fourfold beginning from the fifth week of cultivation. Within these colonies, differentiation of hormogonia also occurred, but no taxis towards the rauwolfia callus or in the opposite direction and, consequently, no daughter colony formation were observed.

As noted above, in this variant of cultivation, cyanobacteria had no specific morphological features at the early stages (16 days). However, after three to four weeks of incubation, the population heteromorphism became much more pronounced to reach its maximum by the end of growth (70 days) (table, Figs. 4d–4g). The proportion of heteromorphic cells comprised 20, 30, and more percent of the total cell number in various specimens. Although most vegetative cells were almost spherical in shape, having a length and width of 4.46 ± 0.19 and 4.14 ± 0.17 μm, some cells measured 2–3 or 6–7 μm. In addition, giant cells 15–18 μm long and 13–15 μm wide, minicells (0.9 by 0.7 μm), and cells of irregular shape were encountered (Figs. 4f, 5), as well as FDCW, which were mostly spheroplasts (Fig. 4e). In all cell types, the sheaths were poorly developed. All cells were embedded in the mucous extracellular matrix. Within the latter, there were zones with low electron density surrounding cells and filled with ultrathin fibrils and fine granular material. A net of more dense framework strands was also distinguishable. Under a scanning electron microscope, the extracellular matrix appeared as a wide-meshed spongy mass (Figs. 4e, 4g).

Thus, the following conclusions can be inferred from this morphological study of *N. muscorum* CALU 304 interacting with the rauwolfia callus tissue: (1) growth of the mixed culture results in spatial integration between cyanobacteria and the plant tissue; specifically structured aggregates are formed, which are periodically renewed; (2) the cyanobacterial morphology varies depending on the zone of their localization in the mixed aggregate; (3) cyanobacterial heteromorphism increases with time

during prolonged associated growth; (4) the fact that in monoculture cyanobacterial heteromorphism does not increase, whereas it is pronounced, after approximately the same time interval, in the mixed culture (irrespective of whether cyanobacteria directly contacted the rauwolfia tissue or not), suggests the plant origin of the factors inducing heteromorphic changes in *N. muscorum* and their rapid diffusion in agarized medium.

I believe the heteromorphic cyanobacterial cells revealed in this study are analogous to the forms that, along with protoplasts and spheroplasts, are known to emerge in populations of unbalanced growth and gram-negative bacteria under conditions of unbalanced growth as a result of adaptation for short-term survival under changing environmental conditions [19]. First, in the mixed cultures, the increased heteromorphism in the *N. muscorum* population correlated with its ability to grow under adverse conditions on the unfavorable incubation medium. Second, in the monoculture on the same medium, the proportion of the heteromorphic cells decreased, and this correlated with growth inhibition and death of the culture.

The bacterial forms that appear under conditions of unbalanced growth seem to be the first stage of the L-transformation [19], which was reported to occur in cyanobacteria contributing to natural symbioses [12] and artificial associations with plants and their tissues cultivated in vitro [18, 20].

In my further work, I shall describe the ultrastructural organization of the heteromorphic cyanobacterial cells revealed in the *N. muscorum–R. serpentina* experimental system and discuss the mechanism of their induction.

ACKNOWLEDGMENTS

I am grateful to O.I. Baulina, M.V. Gusev, and T.G. Korzhenevskaya for critically reading the manuscript.

This work was supported by the Russian Foundation for Basic Research (project no. 97-04-48363).

REFERENCES

1. *Handbook of Symbiotic Cyanobacteria*, Rai, A.N., Ed., Boca Raton: CRC, 1990.
2. Vagnoli, L., Margheri, M.C., Allotta, G., and Materassi, R., Morphological and Physiological Properties of Symbiotic Cyanobacteria, *New Phytol.*, 1992, vol. 120, pp. 243–249.
3. Korzhenevskaya, T.G., Lobakova, E.S., Gorelova, O.A., and Gusev, M.V., Reconstitution of Lichen Symbioses and Syncyanoses, *Aktual'nye problemy eksperimental'noi likhenologii v SSSR. Tr. Botanicheskogo instituta im. V.L. Komarova* (Pertinent Problems of Experimental Lichenology. Proc. Komarov Inst. Botany), Golubkova, N.S., Ed., Leningrad: PO-3 Lenuprizdata, 1991, no. 1, pp. 21–29.
4. Johansson, C. and Bergman, B., Reconstitution of the Symbiosis of *Gunnera manicata* Linden: Cyanobacterial Specificity, *New Phytol.*, 1994, vol. 126, no. 4, pp. 643–652.
5. Rodgers, G.A. and Stewart, W.D.P., The Cyanophyta–Hepatic Symbiosis: I. Morphology and Physiology, *New Phytol.*, 1977, vol. 78, no. 2, pp. 441–458.
6. Enderlin, C.S. and Meeks, J.C., Pure Culture and Reconstitution of the *Anthoceros–Nostoc* Symbiotic Association, *Planta*, 1983, vol. 158, no. 2, pp. 157–165.
7. Bonnet, H.T. and Silvester, W.B., Specificity in the *Gunnera–Nostoc* Endosymbiosis, *New Phytol.*, 1981, vol. 89, no. 1, pp. 121–128.
8. Gantar, M., Kerby, N.W., Rowell, P., and Obreht, Z., Colonization of Wheat (*Triticum vulgare* L.) by Nitrogen-fixing Cyanobacteria: I. A Survey of Soil Cyanobacterial Isolates Forming Association with Roots, *New Phytol.*, 1991, vol. 118, pp. 477–483.
9. Gorelova, O.A., Baulina, O.I., Lobakova, E.S., and Korzhenevskaya, T.G., Heteromorphism of Cyanobacteria in Artificial Associations with Lucerne and Rice, *XIII Vsesoyuz. konf. po elektronnoi mikroskopii (biologiya i meditsina). Tez. dokl.* (Proc. 13th All-Union Conf. on Electron Microscopy (Biology and Medicine)), Moscow, 1988, p. 241.
10. Korzhenevskaya, T.G., Baulina, O.I., Gorelova, O.A., Lobacova, E.S., Butenko, R.G., and Gusev, M.V., Artificial Syncyanoses: The Potential for Modeling and Analysis of Natural Symbioses, *Symbiosis*, 1993, vol. 15, pp. 77–103.
11. Gorelova, O.A., Korzhenevskaya, T.G., and Gusev, M.V., Formation and Oriented Spreading of Cyanobacterial Hormogonia in Model Systems with Higher Plant Tissues, *Vestn. Mosk. Univ. Ser. 16: Biol.*, 1995, no. 4, pp. 19–27.
12. Gorelova, O.A., Baulina, O.I., Shchelmanova, A.G., Korzhenevskaya, T.G., and Gusev, M.V., Heteromorphism of the Cyanobacterium *Nostoc* sp., a Microsymbiont of the *Blasia pusilla* Moss, *Mikrobiologiya*, 1996, vol. 65, no. 6, pp. 824–832.
13. Gorelova, O.A. and Artamonova, K.V., A New Morphological Structure in the Association of Cyanobacteria with Plant Tissue, *Tez. dokl. XIV konf. po elektronnoi mikroskopii (biologiya, meditsina)* (Proc. 14th Conf. on Electron Microscopy (Biology and Medicine)), Moscow, 1992, p. 192.
14. Korzhenevskaya, T.G., Gorelova, O.A., Baulina, O.I., and Gusev, M.V., Accumulation of Reserve Polymers by *Nostoc muscorum* CALU 304 Cells Grown in Mixed Culture with Plant Tissue, *Mikrobiologiya*, 1999, vol. 68, no. 2, pp. 191–197.
15. Gorelova, O.A., Baulina, O.A., and Korzhenevskaya, T.G., Peculiarities of Cell Division in *Nostoc muscorum* CALU 304 in Monoculture and Associations with Plant Tissues, *Mikrobiologiya*, 1999, vol. 68, no. 4, pp. 528–533.
16. Stanier, R.Y., Kunisava, R., Mandell, M., and Cohen-Bazire, G., Purification and Properties of Unicellular

Blue-Green Algae (Order *Chroococcales*), *Bacteriol. Rev.*, 1971, vol. 35, pp. 171–205.

17. Allen, M.B. and Arnon, D.I., Studies on Nitrogen-fixing Blue-Green Algae: I. Growth and Nitrogen Fixation by *Anabaena cylindrica* Lemm., *Plant Physiol.*, 1955, vol. 30, pp. 366–372.
18. Baulina, O.I., Agafodorova, M.N., Korzhenevskaya, T.G., Gusev, M.V., and Butenko, R.G., Cyanobacteria in Artificial Association with the Callus Tissue of Tobacco Plants, *Mikrobiologiya*, 1984, vol. 53, no. 6, pp. 997–1002.
19. Prozorovskii, S.V., Zigangirova, N.A., Konstantinova, N.D., and Kats, L.N., The Phenomenon of Unbalanced Growth in Bacteria, *Mikrobiol. Epidemiol. Immunobiol.*, 1987, no. 12, pp. 94–101.
20. Gorelova, O.A., Baulina, O.I., and Korzhenevskaya, T.G., Ultrastructure of Cyanobacteria Developing in Lucerne Leaf Tissue upon Infection, *IV Resp. konf. po elektronnoi mikroskopii. Elektronnaya mikroskopiya i sovremennaya tekhnologiya. Sb. tezisov* (Proc. 4th Republican Conf. on Electron Microscopy), Kishinev, 1990, pp. 50–51.