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**EXPERIMENTAL ARTICLES**

# Cyanobacterial–Bacterial Complexes in Plant Syncyanoses

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**Abstract**—The morphology and ultrastructure of associative microsymbiont complexes (AMC) isolated from the ferns *Azolla pinnata* and *Azolla* sp. and the apogeotropic roots of the cycad *Cycas revoluta* were studied. The composition of the AMC obtained includes the cyanobionts (symbiotic cyanobacteria) and satellite bacteria (SB). It was found that two types of cyanobacteria that substantially differ in their morphological organization are likely present as cyanobionts in the coralloids of *C. revoluta*. The isolated cyanobiont strains exhibited the morphological traits and regularities of development typical of the genus *Nostoc*; they were characterized by the ability of their cells to divide in mutually perpendicular planes. When isolating AMC from different morphological zones of *C. revoluta* apogeotropic roots, SB growth was revealed only around the pieces corresponding to the coralloid apical zone. No AMC components were revealed around the segments of the basal growth zone. Pure cyanobiont cultures were obtained from the AMC of *C. revoluta* coralloids. The AMC isolated from the ferns *A. pinnata* and *Azolla* sp. are characterized by obligate mutual dependence of the partners (the cyanobiont and SB).

*Key words*: syncyanosis, associative microsymbiont complex, cyanobacteria, satellite bacteria.

The formation of natural plant syncyanoses usually occurs de novo in every generation of the host plant and includes the stage of primary infection of its tissues and organs by cyanobacteria [1]. Problems of the generic and species affiliation of a cyanobiont in each particular syncyanosis are disputable [2, 3]. On the one hand, this is due to the fact that, upon infecting plant tissues and cells, cyanobacteria undergo significant morphological and physiological changes [1–3]. On the other hand, in a number of cases, symbiotic cyanobacteria are represented in a plant by several populations [4] that demonstrate different properties, or are even represented by several species [5, 6]. Natural populations of the same species of cyanobacteria in spatially distant habitats are usually characterized by structural and morphological differences; the variety of clones isolated from one cyanobacterial population testifies to its heterogeneity and may be indicative of the adaptation of cyanobacteria to a diverse and ever-changing habitat [7]. Morphological mutants of cyanobacteria often develop not only upon exposure to various physical factors, but also due to the action of bacterial, fungal, and plant metabolites [7]. The geography of higher plant species that form symbioses with cyanobacteria embraces virtually all of the continents [1], which may be the cause of the diversity of the composition of aboriginal soil microflora, including cyanobacteria.

Primary isolates of cyanobacteria from host plants almost always contain bacteria [6, 8]. The involvement of bacteria and possibly fungi in the formation of the cyanobacteria–cycad symbioses has been suggested [9, 10]. The presence of satellite bacteria at all stages of the development of the *Azolla–Anabaena azollae* symbiosis testifies to its three-component nature and the certain role of *Arthrobacter* bacteria in its stable existence [3, 11].

The process of the isolation of microsymbionts from natural symbioses includes, as a rule, the use of physical methods for the removal of the concomitant microflora (exposure to γ-radiation, UV rays, antibiotics, and low temperature). The use of such methods may result in the selection of minor subpopulations or mutant forms of cyanobionts (thus hindering the cyanobiont) identification and to eliminate satellite bacteria. Most of the isolates of symbiotic cyanobacteria studied differ considerably in the complex of their properties from newly isolated symbionts [12].

The aim of this work was to obtain and characterize the associative microsymbiont cultures involved in the syncyanoses with the ferns *Azolla pinnata* and *Azolla* sp. and the cycad *Cycas revoluta*.

## MATERIALS AND METHODS

Associative microsymbiont cultures (AMC) involved in the syncyanoses with the ferns *Azolla* sp. and *A. pinnata* and with the apogeotropic roots (coralloids) of *C. revoluta* and consisting of symbiotic cyanobacteria (cyanobionts) and satellite bacteria (SB) were the subjects of our study. The AMC from natural syncyanoses were isolated using the technique of multiple inoculations of solid and liquid nutritive media with serial dilutions [13]. Solid media contained 1% agar.

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The primary homogenate of *Azolla* sp. was obtained from H. Gering (Humboldt University, Berlin). *A. pinnata* plants were obtained from the Bakh Institute of Biochemistry of the Russian Academy of Sciences, and the geotropic roots of *C. revoluta* were obtained from plants grown in the greenhouse of the Timiryazev Agricultural Academy. To obtain homogenates from *A. pinnata* plants and the apogeotropic roots of *C. revoluta*, the experimental material was preliminarily washed in warm running water for 30 min and then sterilized with 30% hydrogen peroxide for 20 min. Material contaminated with hydrogen peroxide was washed three times with sterile water, reduced to small fragments with a razor, and placed in glass homogenizers with 1–2 ml of BG-11 mineral medium or its nitrogen-free analog BG-$11_0$ [14]. The completeness of the superficial sterilization of the initial plant material was controlled by inoculating nutrient agar (NA) and the modified Yamada–Kamagata medium with water used for washing the *C. revoluta* apogeotropic roots and the *A. pinnata* plants [15]. The homogenates obtained were resuspended in 5 ml of the cultivation medium and used for the isolation of AMC.

*C. revoluta* AMC were also obtained from pieces of apogeotropic roots. To do this, preliminarily sterilized coralloid roots were cut into 2– to 3-mm fragments with a sterile scalpel and placed into 50-ml flasks with 20 ml of BG-$11_0$, BG-11, and KM [16] media and in petri dishes with the same solid media. After 35 to 45 days of incubation of the material in a luminostat at 20 to 26°C and continuous illumination (1700 lx), primary AMC growth was visually observed as dark green flakes or scales in the liquid media and, on the surface of solid media, as colonies with a coloration characteristic of cyanobacteria. The suspensions obtained were subcultivated in flasks and on petri dishes under the same conditions. When cultivating the AMC on the petri dishes, reinoculations were carried out every 3–5 days using cyanobacterial filaments maximally removed from the primary colony [13].

The AMC were subcultivated once a month (the inoculum dose was 10%) on BG-11 and BG-$11_0$ media supplemented with 5 g/l of sucrose.

The cyanobionts were generically identified by their cultural, morphological, and physiological parameters using *Bergey's Manual* [17].

The number of vegetative cells and heterocysts was counted based on their morphological differences using a Laborlux D (Leitz) microscope.

The specimens for electron microscopy were prepared as described earlier [18].

## RESULTS AND DISCUSSION

The growth of AMC was visually observed after 35 to 45 days of incubation of the fragments of *C. revoluta* apogeotropic roots and *Azolla* sp. and *A. pinnata* homogenates in liquid BG-$11_0$ medium.

**Peculiarities of AMC growth.** When inoculating solid nutritive media with primary AMC isolates from *Azolla* sp., *A. pinnata*, and *C. revoluta*, we observed the formation of primary cyanobiont colonies in the first three days from which hormogonia (movable filaments) appeared and spread in the centrifugal direction over a distance of up to 15 mm. The cessation of the hormogonium movement coincided with the beginning of the formation of secondary colonies. In this process, rod-shaped clusters up to 2 mm in diameter consisting of densely packed spiral trichomes enclosed in a common mucous sheath originated from immovable filaments (Fig. 1). Every five to seven days, repeated formation of hormogonia from secondary colonies occurred with the result that the entire agar surface was covered with cyanobacteria. A pure culture of the AMC cyanobiont isolated from *C. revoluta* coralloids was obtained by transferring the hormogonia maximally removed from the primary colony to a fresh nutritive medium [13].

We did not observe visible growth of SB when growing the AMC obtained from *A. pinnata* and *Azolla* sp. homogenates on NA or on solid mineral media with the addition of organic substrates (sugars, vitamins, casein hydrolysate) for three weeks. However, an electron microscopic study of the cultivated AMC samples showed the SB to be in contact with the cyanobacterial cells (Fig. 2). As a rule, the bacterial cells are situated in close proximity to the envelopes of heterocysts (Figs. 2, 3) and clusters. SB growth was also revealed in the areas of mineral agarized media conditioned with cyanobacterial metabolites along the pathway of the centrifugal movement of hormogonia, seen as white tracks after prolonged (more than 30 days) AMC cultivation.

The growth of the AMC isolates isolated from ferns homogenates on solid media in the form of cyanobiont colonies was accompanied by an insignificant increase in their diameter, and the initially well-defined colony margins gradually became blurred due to the formation of a large amount of lustrous mucus on their surface. Prolonged growth of AMC on the cultivation medium (more than 60 days) was accompanied not only by the vigorous formation of mucus on the surface of the colonies but also by their decolorization. The latter changed color from dark green to yellow–cream, which was due to the growth of SB. However, one week after transferring the fragments of the yellow–cream colonies to a fresh solid mineral medium, we observed the resumption of the characteristic cyanobiont growth described above; i.e., during prolonged cultivation of the isolated associative complexes, periodic replacement of the dominant component was observed. It should be noted that the growth of the cyanobacterial constituent of the complex, the cyanobiont, was always primary, while SB always developed in the portions of the media conditioned with cyanobacterial metabolites or on the colonies of cyanobacteria when they degraded. The AMC isolated from the ferns *A. pinnata* and *Azolla* sp. seem to be composed of the minor com-

ponent of the cyanobionts and their obligate SB. It is known that the predominant AMC cyanobiont from the *Azollae* ferns is incapable of growing outside the host or as a symbiont in association with other symbiotrophic plants [3, 4].

In nature, the ferns of the genus *Azollae* form an obligate symbiosis (the only one among other plant syncyanoses) in which AMC transmission occurs at the sex stage in the life cycle of the host plant [11]. The long joint evolution of the host plant and AMC seems to have determined the high specificity of interaction of the partners in this symbiosis.

Thus, in the AMC obtained from the *Azolla* ferns and the apogeotropic roots of *C. revoluta*, the character of the cyanobiont–SB interaction is different. The AMC isolated from the fern homogenates are characterized by an obligate dependence of partners manifesting itself in their periodic balanced growth and the inability to obtain a pure cyanobiont culture using a complex of microbiological cultural methods of purification.

**The morphology and ultrastructure of AMC cyanobionts.** The growth of cyanobionts on solid mineral media (45 days) results in the formation of compact high colonies with a clearly defined margin. *Azolla* sp. and *A. pinnata* isolates formed lustrous dark green granular colonies, while *C. revoluta* isolates formed larger dull, uneven, brownish green colonies. In larger mature colonies, the number of clusters decreased, while the proportion of filamentous forms (trichomes) often forming compact balls of filaments increased. Further cultivation did not result in an increase in the colony diameter. Prolonged subcultivation of the isolated cyanobionts on agarized media (up to 3 years) did not change the general pattern of isolate growth.

When the cyanobionts were subcultivated in liquid media, the predominant form of their morphological organization was bundles of straight, similarly oriented filaments and very long (more than 100 cells) single twisted filaments.

Two strains of cyanobacteria significantly differing in their morphological organization were isolated from the fragments of the apogeotropic roots of *C. revoluta*. Strain f-1 is characterized by a morphology typical of the genus *Nostoc*. Its trichomes consist of vegetative cells and large lemon-shaped heterocysts exceeding the size of the vegetative cells almost twofold (Fig. 4). The main morphological structure of the other cyanobiont, strain f-2, isolated from *C. revoluta* is large clusters consisting of small cells (Fig. 5). On rupture, the clusters produce short chains consisting of small-sized cells.

The anatomical study of the distribution of microsymbionts in *C. revoluta* coralloids showed the presence of symbiotic cyanobacteria in both the intercellular space of the mucous zone of the cortical parenchyma and in the parenchymatous cells [10]; the population of cyanobacteria located intracellularly significantly differed morphologically from the extracellular population. Cyanobacteria belonging to three genera—*Nostoc*, *Anabaena*, and *Calothrix*—were isolated from sterilized apogeotropic roots of the cycad *Encephalartos trasvenosus*, and cyanobacteria belonging to the genera *Nostoc* and *Anabaena* were isolated from *Macrozamia communis* [5, 6]. The earlier described morphological peculiarities of the structure of cyanobacteria in the coralloids and in the cultures of the cyanobiont strains f-1 and f-2 isolated from the apogeotropic

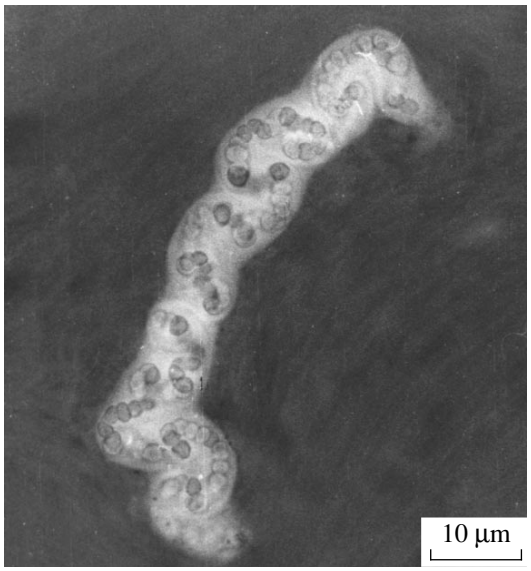


**Fig. 1.** Elongated clusters of the AMC cyanobiont of *Azolla pinnata*.

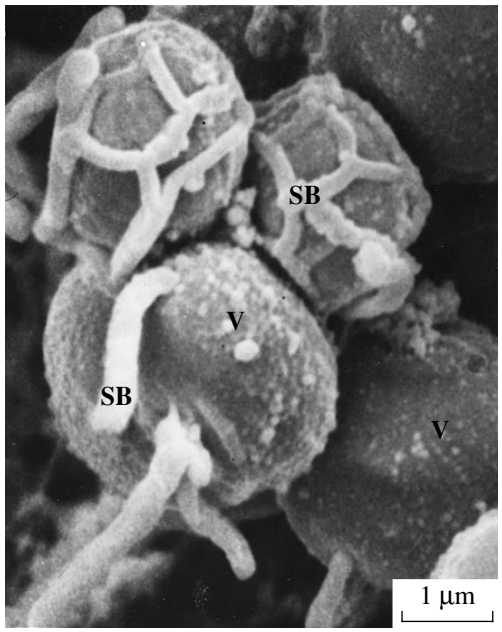


**Fig. 2.** Fragment of the AMC isolated from *Azolla pinnata* ferns. V is a vegetative cyanobiont cell, SB denotes satellite bacteria.

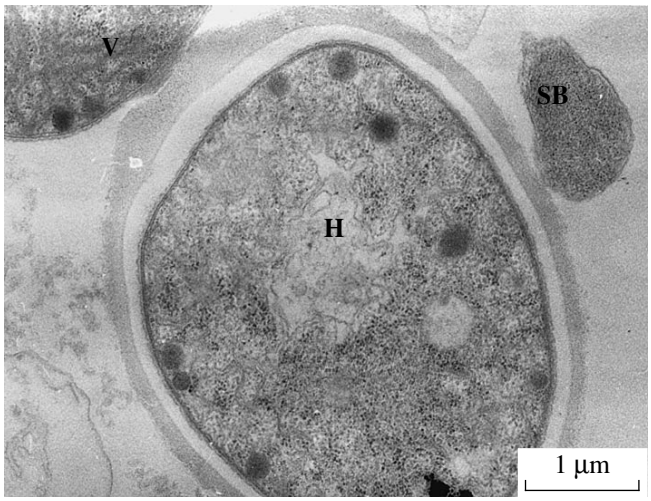


**Fig. 3.** Fragment of the *Azolla* sp. cyanobiont trichome. H is a heterocyst, V is a vegetative cell, and SB denotes satellite bacteria.

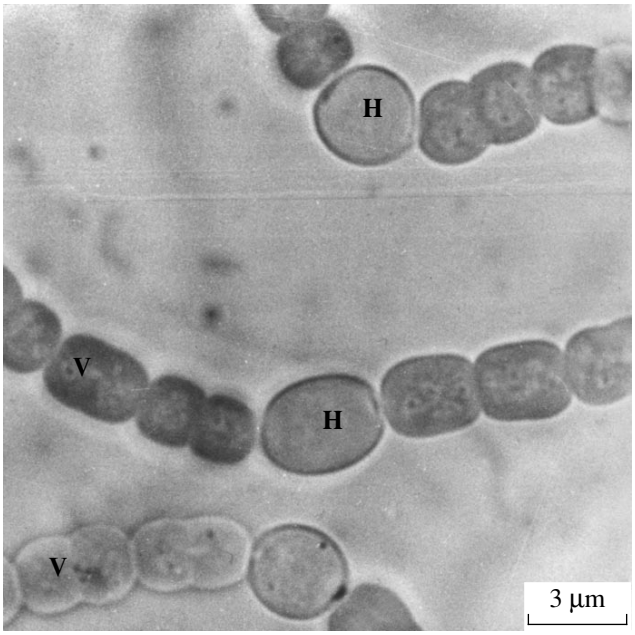


**Fig. 4.** Trichomes of the cyanobiont (strain f-1) isolated from *C. revoluta* coralloids. V marks vegetative cells and H denotes heterocysts.

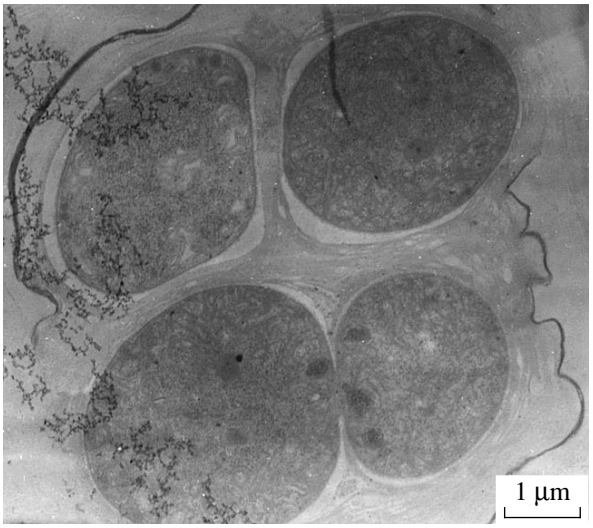


**Fig. 5.** Ultrathin section of a cluster formed by the *Cycas revoluta* cyanobiont (strain f-2).

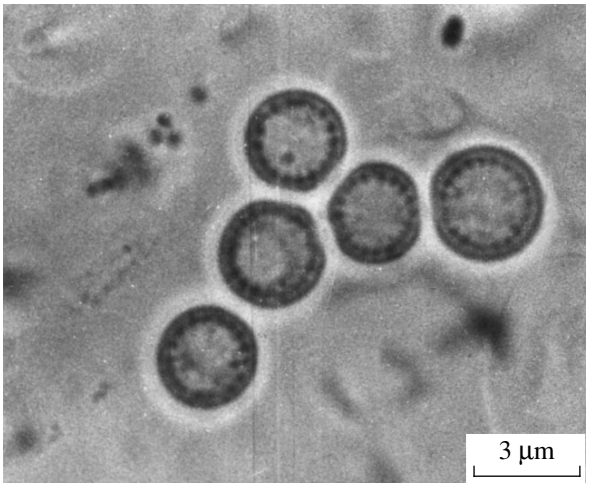


**Fig. 6.** Fragment of a trichome of the *Cycas revoluta* cyanobiont (strain f-1). Vegetative cells divide in mutually perpendicular planes.

roots of *C. revoluta* suggest that two distinct species of cyanobacteria may be present as cyanobionts in the plant under study.

The cyanobiont strains isolated from *Azolla* sp. and *A. pinnata* did not exhibit significant morphological differences from strain f-1 isolated from *C. revoluta* coralloids; they were also characterized by the morphological characteristics typical of the *Nostoc* representatives. There are no clear differences between the genera *Noctoc* and *Anabaena* in the present-day classifications of cyanobacteria, which are, as a rule, based on the complex of morphological characteristics and the regularities of culture development [17]. Many authors propose to regard symbiotic cyanobacteria isolated from the *Azolla* ferns as *Nostoc* species [17].

The characteristic feature of the isolated and cultivated strains of cyanobacteria, except strain f-2, is the high proportion of heterocysts in the total cell number. Under conditions most favorable for heterocyst formation (medium BG-$11_0$ containing 0.5% fructose when cultivating the AMC from *Azolla* and 1% sucrose when cultivating cyanobionts from *C. revoluta*), the proportion of heterocysts constituted 10 and 8% of the total

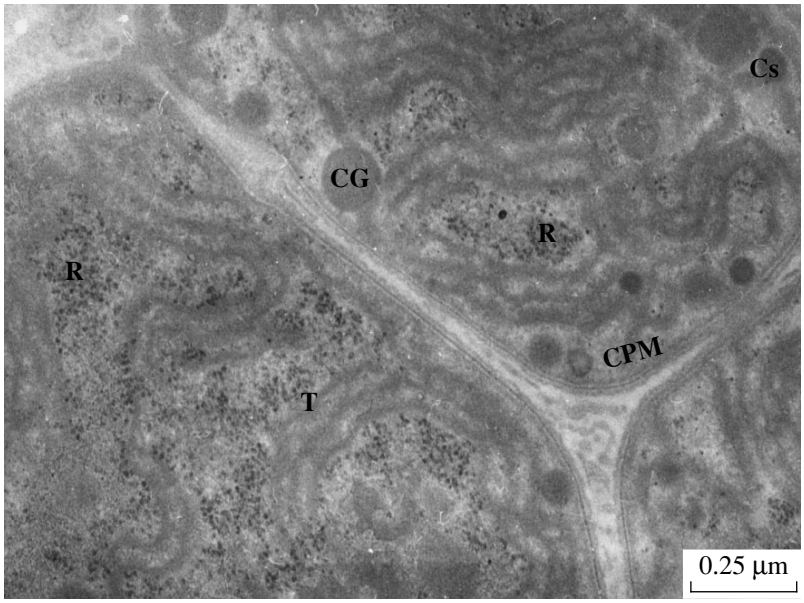


**Fig. 7.** Ultrathin section of the *Azolla* sp. cyanobiont cells dividing in mutually perpendicular planes. T marks thylakoids, R indicates ribosomes, CPM is the cytoplasmic membrane, Cs denotes a carboxysome, and CG marks cyanophycin granules.

cell number, respectively. Heterocysts were often arranged in pairs.

Electron microscopic studies of the isolated microsymbiont cultures showed that the filamentous forms of the cyanobacterial population consisted of vegetative cells and heterocysts, while the population represented by clusters consisted of vegetative cells and protoplasts; the cyanobacterial cells forming clusters were capable of dividing in mutually perpendicular planes (Figs. 6, 7). A similar phenomenon was also described for an in vitro cultivated *Nostoc* sp. cyanobiont isolated from the moss *Blasia pusilla* [19]. The proportion of clusters in the cyanobacterial isolated cultures did not exceed 1.5% of the trichome number and was constantly retained during subcultivation.

**Isolation of AMC from different fragments of *C. revoluta* apogeotropic roots.** Taking into consideration the earlier obtained data on the topography of the distribution of microsymbionts in the coralloids of the cycads *C. revoluta* and *Encephalartos horridus* [10], we tried to isolate *C. revoluta* microsymbionts from the fragments of the apogeotropic roots corresponding to the apical zone from the cyanobacterial localization zone, and from the basal coralloid zone. When the coralloid fragments were placed on nutritive media, SB growth was visible only around the apical zone pieces. The characteristic cyanobiont growth always showed up around the apogeotropic root fragments corresponding to the zone of localization of cyanobacteria, and in a number of cases insignificant cyanobiont growth was observed around the segments of the apical zone. This is likely to be due to the fact that the division of coralloids into zones is conventional and that there are no well-defined boundaries between the apical zone and the cyanobacterial localization zone. The basal coralloid zone is characterized by a more clear-cut morphological boundary, since it has a characteristic pink coloration related to the accumulation of specific phenolic compounds in specialized cells called idioblasts [20]. No growth of the AMC components was revealed around the segments of the basal part of the coralloid.

Earlier, it was suggested that symbiotrophic plant species possess mechanisms for regulating the spreading, growth, and physiological activity of microsymbionts in the host tissues [1–3]. According to our data, the amount and the arrangement of idioblasts containing phenolic compounds exhibit significant variation from the apex of the coralloid to its basal part [21]. Phenolic compounds play a great role in plant pathology, since they are able to inhibit the growth of the bacteria and fungi present in host plant tissues or considerably decrease their growth rate [22].

The sensitivity of microorganisms to individual phenolic compounds is different, which seems to account for the existence of growth zones of both bacteria and cyanobacteria within the apogeotropic root and to testify to the control of the infection process and the topography of the AMC components in coralloids by the plant.

To conclude, the growth of cyanobacteria in natural syncyanoses is controlled by the host plant. The cyanobionts present in the internal cavities or cells of a plant are exposed to the action of its metabolite complex. The bacteria, as a minor AMC component, seem to play an essential role both in the processes of formation and stable existence of plant syncyanoses.

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