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EXPERIMENTAL ARTICLES

Associative Cyanobacteria Isolated from the Roots of Epiphytic Orchids

E. A. Tsavkelova*, [1], E. S. Lobakova, G. L. Kolomeitseva***,**
T. A. Cherdyntseva*, and A. I. Netrusov*

**Department of Microbiology, Biological Faculty, Moscow State University, Vorob'evy gory, Moscow, 119899 Russia*

***Department of Cell Physiology and Immunology, Biological Faculty, Moscow State University, Vorob'evy gory, Moscow, 119899 Russia*

****Central Botanical Garden, Russian Academy of Sciences, Moscow, 127276 Russia*

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Abstract—Associative cyanobacteria were isolated from the rhizoplane and velamen of the aerial roots of the epiphytic orchids *Acampe papillosa*, *Phalaenopsis amabilis*, and *Dendrobium moschatum* and from the substrate roots of *A. papillosa* and *D. moschatum*. Cyanobacteria were isolated on complete and nitrogen-free variants of BG-11 medium. On all media and in all samples, cyanobacteria of the genus *Nostoc* predominated. *Nostoc*, *Anabaena*, and *Calothrix* were isolated from the surface of the *A. papillosa* aerial roots, whereas the isolates from the substrate roots were *Nostoc*, *Oscillatoria*, and representatives of the LPP group (*Lyngbia*, *Phormidium*, and *Plectonema*, incapable of nitrogen fixation). On the *D. moschatum* substrate roots, *Nostoc* and LPP group representatives were also found, as well as *Fischerella*. On the aerial roots of *P. amabilis* and *D. phalaenopsis* grown in a greenhouse simulating the climate of moist tropical forest, cyanobacteria were represented by *Nostoc*, LPP group, and *Scytonema* in *D. phalaenopsis* and by *Nostoc*, *Scytonema*, *Calothrix*, *Spirulina*, *Oscillatoria*, and the LPP group in *P. amabilis*. For *D. moschatum*, the spectra of cyanobacteria populating the substrate root rhizoplane and the substrate (pine bark) were compared. In the parenchyma of the aerial roots of *P. amabilis*, fungal hyphae and/or their half-degraded remains were detected, which testifies to the presence of mycorrhizal fungi in this plant. This phenomenon is attributed to the presence of a sheath formed by cyanobacteria and serving as a substrate for fungi.

Key words: epiphytic orchids, sheath, associative cyanobacteria.

Epiphytic, mostly tropical, orchid species are adapted to growth on high foliaceous trees, where they gain more light and are more competitive. They attach to the plant bark, which serves as a support, and form numerous aerial roots, whose surface is a favorable habitat for phototrophic and diazotrophic microorganisms.

We have previously revealed cyanobacteria not only on the root surface but also in the root velamen of tropical orchid species [1, 2]. Both the quantitative and qualitative composition of the associative cyanobacteria depended on the orchid growth conditions. In plants grown in a greenhouse under conditions simulating a moist tropical climate, the population density of the aerial phototrophic microorganisms associated with roots was so high that a dense dark green sheath 1–3 mm thick was formed on the entire root surface [2]. The filamentous cyanobacteria interlaced with fungal hyphae, forming the peculiar framework of the sheath, the interstices of which were filled with unicellular phototrophs and bacteria.

In our previous work [1], we also showed that the species composition of cyanobacteria isolated from the surface of aerial roots of the epiphytic species *Dendrobium moschatum* differed from that of cyanobacteria isolated from the surface of underground roots of the ground species *Calanthe vestita* var. *rubro-oculata*: only *Nostoc* representatives were characteristic of *D. moschatum*, whereas both *Nostoc* and *Oscillatoria* occurred on the roots of *C. vestita* var. *rubro-oculata*.

It should be mentioned here that, in lichen, both filamentous cyanobacteria (*Nostoc*, *Scytonema*, *Calothrix*, and *Fischerella*) and unicellular forms (*Gloeothece*, *Gleocapsa*, and *Synechocystis*) may be the symbiontic partners of fungi [3], whereas only filamentous cyanobacteria, mostly those belonging to the genus *Nostoc*, occur in symbioses with higher plants (cited from [4]). In addition, in plant syncyanoses, cyanobionts function as a nitrogen-fixing component, whereas in symbioses with fungi, cyanobacteria perform both nitrogen fixation and photosynthesis.

The aim of this work was isolation and identification of cyanobacteria populating the rhizoplane of substrate roots and the surface and velamen of aerial roots of tropical epiphytic orchid species.

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[1] Corresponding author (e-mail: tsavkelova@mail.ru).

Associative cyanobacteria isolated from the root surface of epiphytic orchids

Orchid species	Medium	
	nitrogen-free BG_0-11	complete medium BG-11
	Substrate roots	
D. moschatum	*Nostoc*	*Nostoc*, LPP, *Fischerella*
A. papillosa	*Nostoc*	*Nostoc*, *Oscillatoria*, LPP
	Aerial roots	
A. papillosa	*Nostoc*, *Anabaena*	*Nostoc*, *Anabaena*, *Calothrix*
*D. phalaenopsis**	*Nostoc*, LPP, *Scytonema*	*Nostoc*, LPP, *Scytonema*
*P. amabilis**	*Nostoc*, *Scytonema*, *Calothrix*, LPP and cyanobacteria of section 1	*Nostoc*, *Scytonema*, *Calothrix*, LPP, cyanobacteria of section 1, *Spirulina*, *Oscillatoria*

* Species characterized by the formation of a sheath on the root surface.

MATERIALS AND METHODS

This study used generatively mature epiphytic orchids, specifically, aerial and substrate roots of *Acampe papillosa*, substrate roots of *Dendrobium moschatum*, and aerial roots of *Phalaenopsis amabilis* and *Dendrobium phalaenopsis*. The plants were obtained from the Collection Greenhouse of the Central Botanical Garden, Russian Academy of Sciences, Moscow. The conditions of orchid growth and sampling of experimental material were described earlier [1, 2]. The substrate used for growing the epiphytic orchid *D. moschatum*, pine bark, was also studied.

To isolate cyanobacteria, 1 g of the roots was twice washed in 100 ml of sterile tap water on a shaker (120 rpm) for 10–15 min. The roots were cut up into fragments 5 mm long with a sterile razor and mixed to homogenize the sample. From 15 to 20 root fragments were placed into 50-ml flasks containing 30 ml of either BG-11 medium or its nitrogen-free analogue BG_0-11 [5]; inoculation was conducted at room temperature and a 700-lx illumination for 1.5 months. To prevent fungal growth, nystatin (50 mg/ml) was added to the medium. Pine bark fragments were incubated as described above on complete nitrogen-containing medium.

Pure cultures were isolated by inoculation of solid media of the same compositions. An aliquot (0.1 ml) sampled with a pipette from an enrichment culture was applied to agarized medium and spread over one or several plates with a spatula to obtain individual colonies, which was possible, in particular, due to self-isolation of motile cultures. Another method used to isolate pure cultures was picking up with a loop an apparently homogeneous colony, a clump, from an enrichment culture and inoculating liquid medium of the same composition; after a period of cultivation, culture aliquots were plated onto solid medium. Since pure cultures of unicellular cyanobacteria are difficult to obtain, they were studied only in enrichment cultures.

Genus-level identification of cyanobacteria was performed based on their cultural, morphological, physiological, and biochemical characteristics according to common standards using the ninth edition of *Bergey's Manual* [6]. The following characteristics were taken into account: the pattern of culture growth on solid and liquid media, pigmentation, the presence of sheaths on trichomes, false or true trichome branching (in filamentous cyanobacteria), occurrence of hormogonia and their motility, vegetative cell morphology in trichomes and hormogonia, the presence and location of akinetes and heterocysts, and nitrogen-fixing ability. More comprehensive identification was not performed, because we did not aim to determine the species affiliation of the isolates. Further cultivation of the nitrogen-fixing cyanobacteria of the order *Nostocales* was carried out in nitrogen-free liquid medium.

To assess the diversity of cyanobacterial forms in pine bark, which was used as the substrate for *D. moschatum* growth, bark fragments were used to inoculate complete nitrogen-containing medium, and the enrichments were incubated as described above.

Microscopic examinations of enrichment and pure cultures of cyanobacteria were performed using a Laborlux D (Germany) light microscope (in this paper, micrographs of the enrichment cultures are presented). To detect hyphae of mycorrhizal fungi in the root interior, sections of aerial roots of *Phalaenopsis amabilis* were examined after fixation and staining by Stowtone's method [7].

RESULTS AND DISCUSSION

This study dealt with several aspects of the development of symbiotic associations of cyanobacteria with epiphytic orchids. To study one of these aspects, we attempted the isolation of cyanobacteria from aerial and substrate roots of a given plant species using media differing in the content of nitrogen (among the plants examined, substrate roots are developed by *A. papillosa*

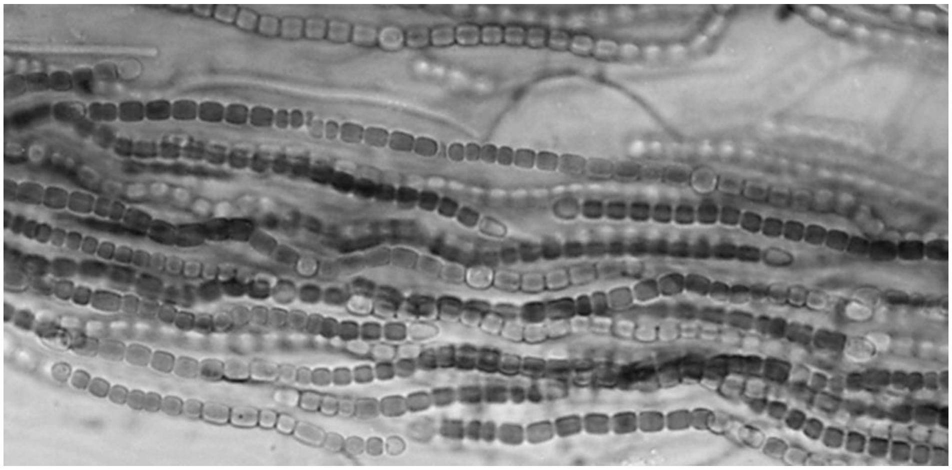

Fig. 1. *Nostoc* (nitrogen-free medium, aerial roots of *P. amabilis*). Light microscopy, 3550×.

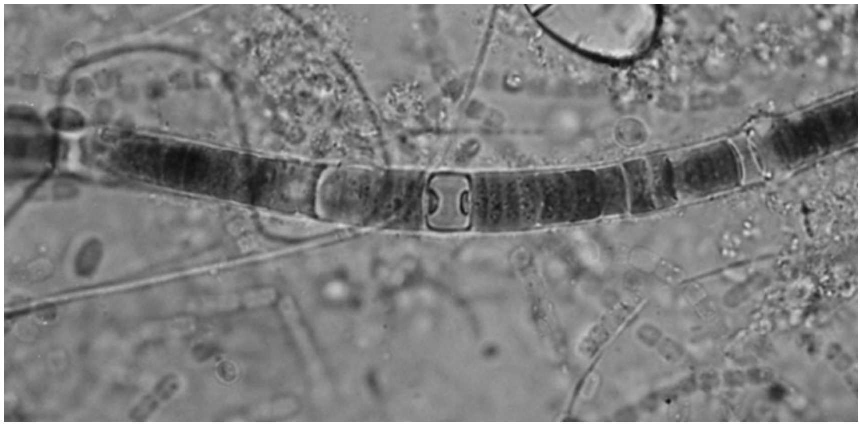

Fig. 2. *Scytonema* (nitrogen-free medium, aerial roots of *P. amabilis*). Light microscopy, 3200×.

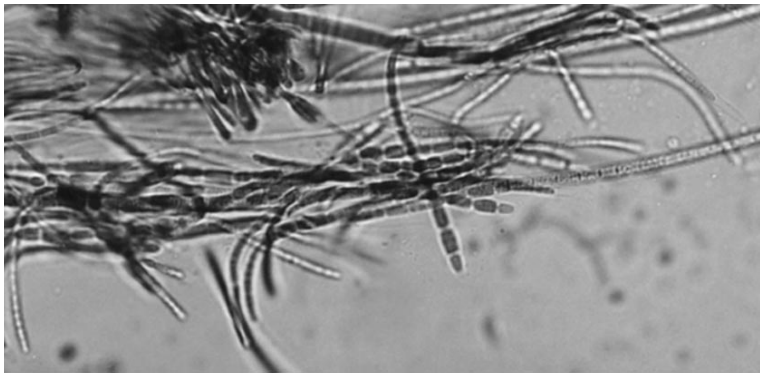

Fig. 3. *Calothrix* (nitrogen-free medium, aerial roots of *P. amabilis*). Light microscopy, 1400×.

and *D. moschatum*). The influence of various climatic zones on the spectrum of cyanobacteria populating aerial roots of orchids was simulated in greenhouses with different temperature and humidity conditions [2].

On the nitrogen-free medium BG_0-11, cyanobacteria of the genus *Nostoc* were isolated from the surface of substrate roots of *A. papillosa*, whereas, from the aerial roots, cyanobacteria of the genus *Anabaena* were also isolated under the same conditions. On the complete medium BG-11, cyanobacteria of the genera *Nostoc* and *Oscillatoria* and of the LPP group (genera incapable of nitrogen fixation: *Lyngbia*, *Phormidium*, and *Plectonema*) were isolated from the substrate roots, and cyanobacteria of the genera *Nostoc*, *Anabaena*, and *Calothrix* were isolated from the aerial roots (table).

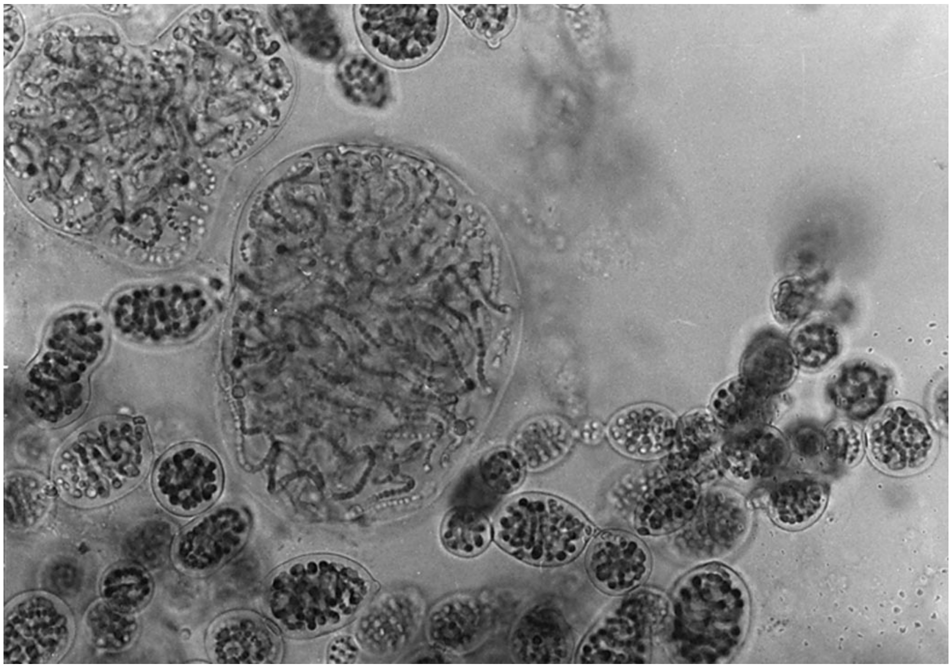

Fig. 4. Clusters of *Nostoc* sp. isolated from the aerial roots of *A. papillosa* on nitrogen-free medium. Light microscopy, 1750×.

In *D. moschatum* grown in the same greenhouse as *A. papillosa*, representatives of the genus *Nostoc* were the only cyanobacteria detected on substrate roots on the nitrogen-free medium BG$_0$-11. In our previous study [1], members of the genus *Nostoc* were also the only isolates from the aerial roots of *D. moschatum* (on both types of media).

Substrate roots develop in orchids when their aerial roots enter substrate (pine bark in our case). After morphological changes, substrate roots function as ordinary soil roots. The substrate used differs from soil in that it promotes the access of air and some light to the roots; at the same time, it retains moisture. Thus, it may serve as a suitable econiche for population by cyanobacteria. It was important to elucidate whether the spectrum of cyanobacteria in the rhizoplane of substrate roots differs from that in the substrate used for the growth of the orchid (we studied this on the example of *D. moschatum*). The spectra of cyanobacteria revealed on BG-11 medium proved to be identical: representatives of the genera *Nostoc* and *Fischerella* and of the LPP group were isolated from pine bark and from the rhizoplane of the substrate roots. This can be explained by the fact that both the plant and its substrate occurred under similar environmental conditions (temperature, humidity, availability of microelements and light). The only distinction between the enrichments was the following: after cultivation for 2 months, the enrichment culture obtained from the substrate was abundant in *Fischerella* trichomes, whereas cyanobacteria of the genus *Nostoc* were the major isolates from the enrichment culture obtained from substrate roots, and the colonies of *Fischerella* were scarce. This assessment, qualitative rather than quantitative, testifies nevertheless to the fact that *Fischerella* prefers to colonize the pieces of pine bark (substrate) rather than to enter associative symbiosis with the orchid plant. It should be noted that *Fischerella* failed to be isolated from the substrate roots of *D. moschatum* on nitrogen-free

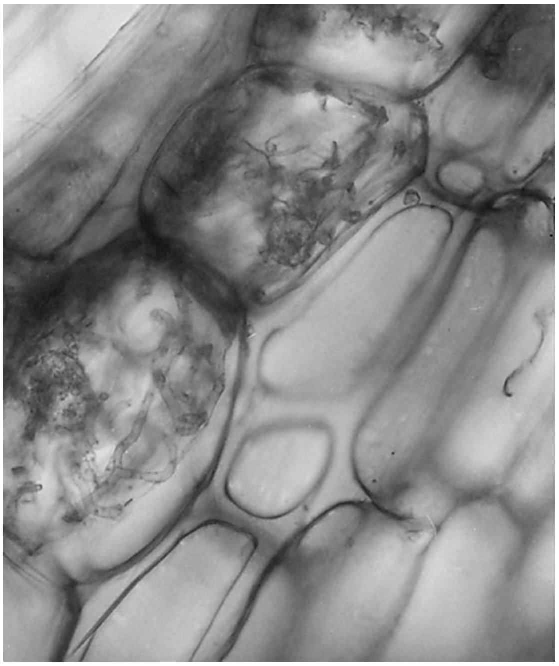

Fig. 5. Hyphae of *P. amabilis* within the plant root tissues (parenchyma). Light microscopy (differential staining by Stowtone’s method), 3150×.

medium, although this species fixes nitrogen and forms heterocysts even on the complete BG-11 medium.

We also studied cyanobacteria constituting the complex structure of the sheath that develops on the aerial roots of *P. amabilis* and *D. phalaenopsis* grown under conditions of a rainy tropical forest (this sheath is described in detail in an accompanying paper [2]). As in the case of *D. moschatum* and *A. papillosa*, the orchids *P. amabilis* and *D. phalaenopsis* grown in the same greenhouse differed in the composition of cyanobacteria. For *D. phalaenopsis*, similar sets of isolates were obtained on media of both types. These were *Nostoc, Scytonema*, and the LPP group (table). Cyanobacteria of the genera *Nostoc, Scytonema*, and *Calothrix* and of the LPP group were isolated from the *P. amabilis* aerial roots on nitrogen-free medium, and unicellular cyanobacteria were obtained in enrichment cultures. On nitrogen-containing medium, *Spirulina* and *Oscillatoria* were also detected along with the aforementioned cyanobacteria.

All filamentous cyanobacterial species of the genera *Anabaena, Nostoc, Scytonema,* and *Calothrix* grown in liquid nitrogen-free medium contained heterocysts both in enrichment and pure cultures (as shown in Figs. 1–3 on the example of a *P. amabilis* enrichment culture). Unicellular cyanobacteria of the first subgroup grew on nitrogen-free medium apparently at the expense of nitrogen-containing metabolic products of the nitrogen-fixing filamentous strains. It is known that soil cyanobacteria incapable of nitrogen fixation utilize extracellular nitrogen-containing products released by nitrogen-fixing cyanobacteria even when the growth medium contains inorganic nitrogen [8]. At the same time, some unicellular and filamentous cyanobacteria can fix nitrogen without heterocyst formation. These are species of the genera *Synechococcus, Gloeothece, Dermocarpa*, and *Oscillatoria* and of the LPP group (cited from [8]).

Nostoc sp. isolated from *A. papillosa* aerial roots formed globe-shaped colonies of various sizes at the initial stage of growth in both enrichment and pure cultures in liquid media with and without nitrogen (Fig. 4). Similar colonies of *Nostoc* were formed in enrichments on liquid nitrogen-containing medium derived from *P. amabilis* aerial roots. These clusters of *Nostoc* trichomes evidently develop due to the jellylike gel produced by *Nostoc* and holding the trichomes together.

The long interlaced aerial roots of *P. amabilis* grown under conditions simulating those of a rainy tropical forest formed a sort of tousled beard. The roots were completely covered by sheaths consisting of a dark green cyanobacterial mass, in which we revealed bacterial cells and their agglomerates as well as fungal hyphae [2]. At the same time, the cyanobacteria that we isolated are known to produce significant amounts of organic substances [9] and to possess a high nitrogen-fixing activity (the nitrogen-fixing activity of the sheaths is described in an accompanying paper [2]). The question therefore arose whether the cyanobacterial sheath formed at the expense of the extracellular slime has an effect on the development of the mycorrhizae-forming symbiotic fungus typical of all orchids. In nature, successful germination of orchid seeds is impossible in the absence of the mycobiont, whereas root mycorrhization is not an obligatory phenomenon, although most orchids proceed in degrading fungal hyphae also when they are generatively mature photosynthesizing plants [10, 11]. At the same time, the mycorrhizae-forming fungus is known to be present in the roots of epiphytic orchids growing within the substrate or in the root portion adjoining the substrate [11, 12]. The photosynthesizing root tissues of the aerial roots do not contain the endophytic fungus; this is characteristic of both wild orchids [12] and orchids grown in greenhouses [11]. We were the first to reveal, by examination of differentially stained sections of large aerial roots of *P. amabilis*, fungal hyphae and/or their half-digested remains in cells of the parenchyma of this plant (Fig. 5); this finding testifies to the presence of the mycorrhizal fungus. In our opinion, aerial roots covered by a dense sheath become incapable of photosynthesis, and the sheath itself may be considered a particular econiche populated by the mycorrhizae-forming fungus, which utilizes as the substrate the slime and extracellular substances released by the associative bacterial community.

Our study is the first to report the isolation of cyanobacteria from aerial and substrate roots of epiphytic orchids. In general, aerial roots are more abundantly populated by cyanobacteria than substrate roots. The aerial roots of orchids grown under conditions of permanently high humidity and temperature, i.e., under conditions typical of tropical forests, are a most favorable econiche for cyanobacteria. The spectrum of associative cyanobacteria isolated from aerial roots of *P. amabilis* and *D. phalaenopsis* is broader than that characteristic of orchids grown in a greenhouse with a drier climate. Cyanobacteria of the genera *Nostoc* and *Anabaena* (the latter is often considered as belonging to the genus *Nostoc* [3, 6]) were invariably isolated from all samples of both substrate and aerial roots of epiphytic orchids. Note that, in most plant syncyanoses, cyanobionts belong to these genera and play the role of nitrogen-fixing diazotrophs [3]. Of special importance is the fact that the root sheath, formed primarily by cyanobacteria, serves as the substrate for the mycorrhizae-forming fungus, whose hyphae penetrate the root.

Further study of physiological and biochemical properties of the cyanobacterial isolates will be helpful for understanding their contribution to the homeostasis of the consortium constituted by the host plant and associated microorganisms (cyanobacteria, bacteria, and fungi). In natural associations of autotrophic cyanobacteria, heterotrophic bacteria, and fungi, equilibrium is maintained, which determines the association stability and extends the ecological areal of each partner [8]. A comprehensive study of plant-associated microorganisms may be helpful for the cultivation of orchids.

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