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EXPERIMENTAL ARTICLES

Localization of Associative Cyanobacteria on 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—This work is the first study of the localization of phototrophic microorganisms in the rhizoplane and velamen of epiphytic orchids, namely, on the aerial and substrate roots of *Acampe papillosa* and *Dendrobium moschatum* and on the aerial roots of *Phalaenopsis amabilis* and *Dendrobium phalaenopsis*. The composition of the bacterial community on the plant roots depended on the conditions of plant growth. Under conditions simulating the climate of moist tropical forests, the aerial roots proved to be populated with phototrophic microorganisms, among which cyanobacteria predominated. Interlaced fungal hyphae and filamentous cyanobacteria formed a sheath on the surface of the aerial roots. The nitrogen-fixing capacity of the sheath of the aerial roots was studied on the example of *P. amabilis.*

Key words: epiphytic orchids, velamen, cyanobacteria, nitrogen fixation.

Tropical species comprise 90–95% of all species of the family Orchidaceae [1], and 70–75% of them are epiphytes, which, for at least a part of their life, inhabit other plants, mostly high trees; however, they are not parasites [2]. The aerial roots of epiphytic orchids are covered with a hygroscopic multilayered sheath of dead cells (velamen). It is a specific ecological niche populated by associative microorganisms [3]. However, the data published on the qualitative and quantitative composition of these associative microorganisms and their possible role in the life of orchids are scarce [4, 5]. At the same time, the term *helper bacteria* has been widely used over the last decade to designate minor components of stable symbiotic associations [6–8]. As a rule, associative microorganisms play an important role in the formation and stable existence of symbioses, as well as in extension of the ecological range of host plant growth [9].

In our previous work, we examined the surface of soil roots of *Calanthe vestita* var. *rubro-oculata* and of the aerial roots of epiphytic *Dendrobium moschatum,* both grown in a greenhouse [3]. Both heterotrophic bacteria and cyanobacteria (varying in their species composition) were revealed in the rhizoplanes. In our opinion, the presence of cyanobacteria among associative microorganisms of epiphytic orchids is of special importance, since these bacteria fulfil a wide range of physiological and biochemical reactions and are capable of rapid switching from one metabolic pathway to another [10]. In natural symbioses with autotrophic organisms, cyanobacteria play the role of the diazotrophic component, and in symbioses with heterotrophic organisms, they play the role of autotrophic and diazotrophic components (e.g., in lichens).

A single report on the presence of cyanobacteria in the orchid velamen [4] provided no evidence of the species composition, distribution in the roots, or possible role of these microorganisms.

In this work, we have examined the localization of cyanobacteria on the roots of epiphytic orchids of various species. The nitrogen-fixing activity of associative microorganisms was also studied.

MATERIALS AND METHODS

Both aerial and substrate roots of the epiphytic orchids *Acampe papillosa* and *Dendrobium moschatum,* as well as the aerial roots of *Phalaenopsis amabilis* and *Dendrobium phalaenopsis,* grown under conditions of the Collection Greenhouse of the Central Botanical Garden, Russian Academy of Sciences (Moscow), were the subject of our study. All plants were generatively mature.

The first two orchids were grown as pot plants, using osmunda rhizome and sphagnum moss (3 : 1) or pine bark as the substrates for *D. moschatum* and pine bark as the substrate for *A. papillosa.* In the greenhouse, air humidity was 60 ± 10%; when the growth

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

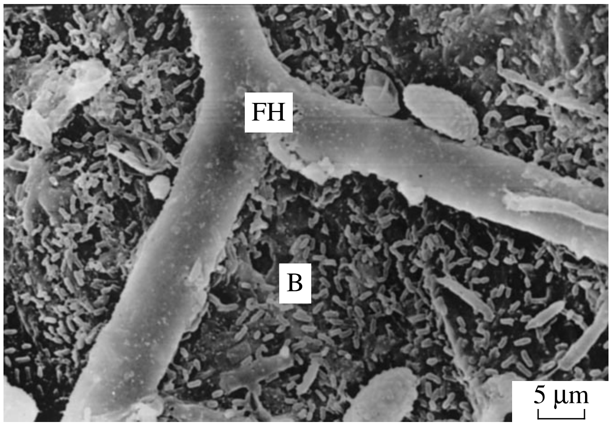

Fig. 1. Fragment of the *A. papillosa* aerial root surface. Scanning electron microscopy. B, bacteria; FH, fungal hyphae.

slowed down, the plants needed minimal watering and cool and dry maintenance. Conversely, *P. amabilis* and *D. phalaenopsis,* grown under conditions of constant 90% air humidity at 25–28°C on plexiglass bars (supporting the plants), needed daily watering (sprinkling) at any time of year. Roots that were at least one year old were sampled with sterile forceps and razors and put into sterile flasks. Microorganism location on the orchid roots was studied with a Laborlux D light microscope (Germany) and an Amray 18301 scanning electron microscope (United States).

Sections of native roots and of roots fixed and differentially stained by Stowtone's method [11] were examined under a light microscope. For scanning electron microscopy, specimens were prepared as described previously [3].

Potential nitrogen-fixing activity (NFA) of the microorganisms associated with the root surface was measured for fragments of the *P. amabilis* aerial roots covered by the thickest sheaths (see below). NFA was measured in penicillin vials by the acetylene method [12] using a Chrom-41 gas chromatograph (Laboratorni pristroje, Czechia). The experiments were run in triplicate. NFA was calculated using a standard formula [12] per gram biomass of the sampled roots.

RESULTS AND DISCUSSION

In nature, the epiphytic orchids studied in this work grow at altitudes from 300 to 2000 m above sea level under conditions of intense illumination and strong air motion. *A. papillosa* grows in Vietnam, India, Bhutan, Burma, Thailand, Laos, and Nepal; *D. moschatum* also occurs in South China, in addition to the aforementioned countries. *P. amabilis* and *D. phalaenopsis* grow in New Guinea; Indonesia; Philippines; and Queensland, Australia.

The natural ecological features of the orchid species chosen by us are especially expressed in the outward appearance of their aerial roots. Thus, *P. amabilis* and *D. phalaeopsis*, growing under conditions of natural or constant artificial 90% air humidity, have the root surface covered almost completely by a dark green mass of phototrophic organisms, forming a peculiar sheath 1–3 mm thick. *A. papillosa* and *D. moschatum,* growing under conditions of 60% air humidity, lack such a thick dark green layer of associative microorganisms on the surface of their aerial roots. The latter two species possess aerial roots with a glossy silvery surface. The surface of *D. moschatum* roots exhibit fibers formed by phototrophic microorganisms, which sometimes form a greenish fibrous film on the larger roots of *A. papillosa*.

The above orchid species contain chloroplasts in the cells of the aerial root tips, which accounts for their

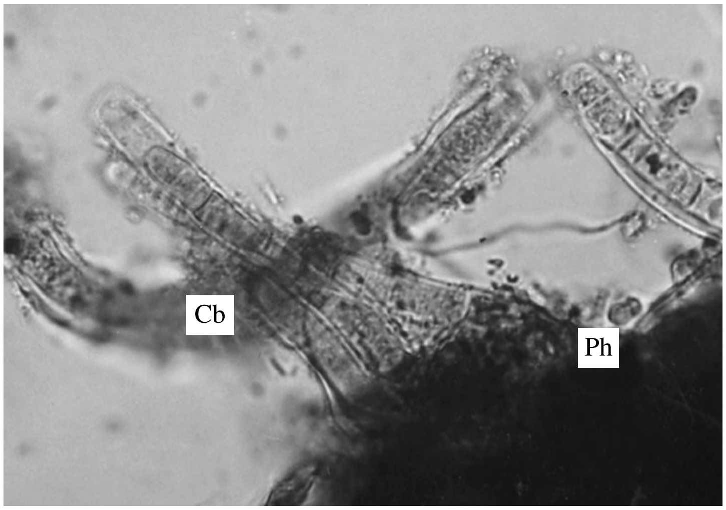

Fig. 2. Fragment of the *P. amabilis* aerial root surface. Light microscopy (fixation and staining according to Stowtone). Ph, phototrophic microorganisms; Cb, cyanobacteria; 2800×.

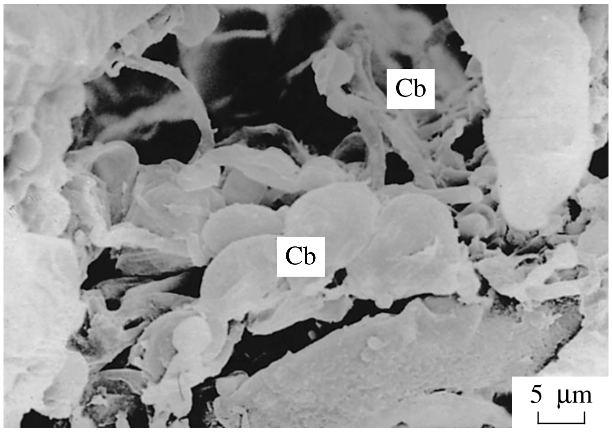

Fig. 3. Fragment of the *P. amabilis* aerial root surface. Scanning electron microscopy. Cb, cyanobacteria.

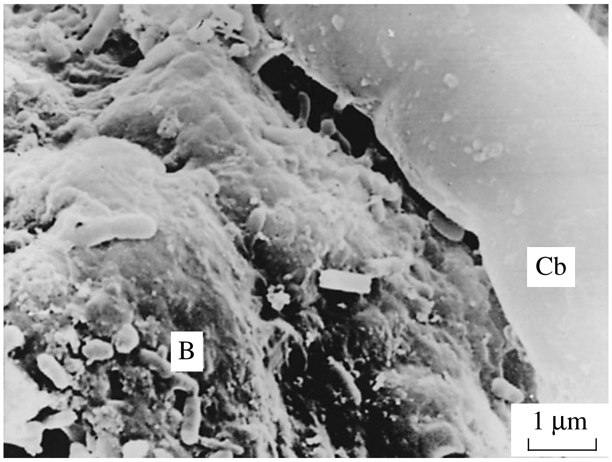

Fig. 4. Fragment of the *P. amabilis* aerial root surface. Scanning electron microscopy. B, bacteria; Cb, cyanobacteria.

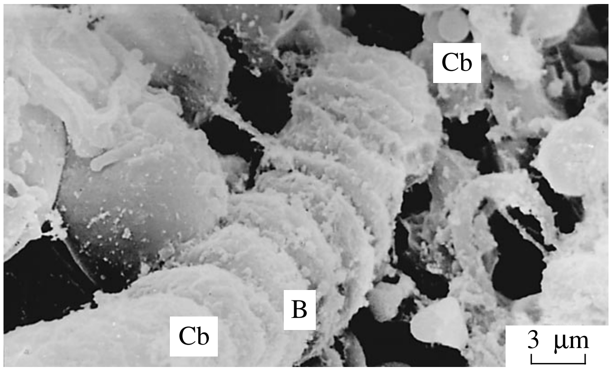

Fig. 5. Fragment of the *D. phalaenopsis* aerial root surface. Scanning electron microscopy. B, bacteria; Cb, cyanobacteria.

light green coloration. The possibility of photosynthesis in the parenchymal cells of the orchid aerial roots has been discussed in the literature, although its importance for the plant development is insignificant [13].

Electron microscopic scanning of the aerial root surface in *A. papillosa* (Figs. 1, 9) and the surface of substrate roots of *A. papillosa* and *D. moschatum* revealed abundant bacterial cells of different morphological types: individual cells and cells united into agglomerates by an intercellular matrix, fungal hyphae, and cyanobacteria. However, comparison of micrographs of surfaces of aerial and substrate roots obtained by scanning electron microscopy showed that, in these orchids, the bacterial population of the aerial root rhizoplane was much more abundant than that of the rhizoplane of substrate roots.

Examination of the surface of aerial roots under a light microscope revealed filamentous cyanobacteria and unicellular phototrophic microorganisms on the root sheaths of *P. amabilis* and *D. phalaenopsis* (Fig. 2). Scanning electron microscopy of the surface of aerial roots of *P. amabilis* and *D. phalaenopsis* also revealed heavy growth of cyanobacteria of various morphotypes. Figures 3 and 4 show *P. amabilis* root fragments carrying on their surface filamentous cyanobacteria of at least two types. The first and the second types were represented by chains of ellipsoid and disk-shaped flattened cells. The surface of aerial roots of *D. phalaenopsis* exhibited similar morphological organization (Fig. 5). In addition to filamentous cyanobacteria, short chains and individual cells similar in size to cyanobacteria occurred between fungal hyphae (Figs. 6, 7). Thus, interlaced filamentous cyanobacteria and fungal hyphae form a peculiar framework of the sheath, the interstices of which are filled with unicellular photosynthesizing microorganisms (cyanobacteria, algae) and bacteria.

In epiphytic orchids, the external layer of the aerial roots, the velamen, plays the following roles: mechanical protection, reflection of solar radiation, preservation of root parenchyma from excessive loss of water, and retaining of mineral salt solutions [14]. The velamen is readily permeable to oxygen and carbon dioxide [15]. A specific feature of a mature velamen is the presence of large perforations that are elliptical slits or holes between secondary bulges of the plant cell walls. After drying of aerial roots, the perforations are enlarged to occupy up to 50% of the total velamen area [16].

The velamen is an econiche suitable for colonization by associative microorganisms, both autotrophic and diazotrophic. By the example of *D. phalaenopsis* and *A. papillosa*, the velamen was shown to lack filamentous cyanobacteria (Figs. 8, 9). At the same time, numerous cells grouped in clusters were found within the velamen and on the surface of the aerial roots (Figs. 6, 9) of the orchids examined, as well as on the aerial roots of *D. moschatum* [3]. They can be considered heterocysts of cyanobacteria as judged from the cell size and the presence of specific pores. Similar morphophysiological changes have been observed in cyanobacteria involved in symbiotic relationships with fungi (in

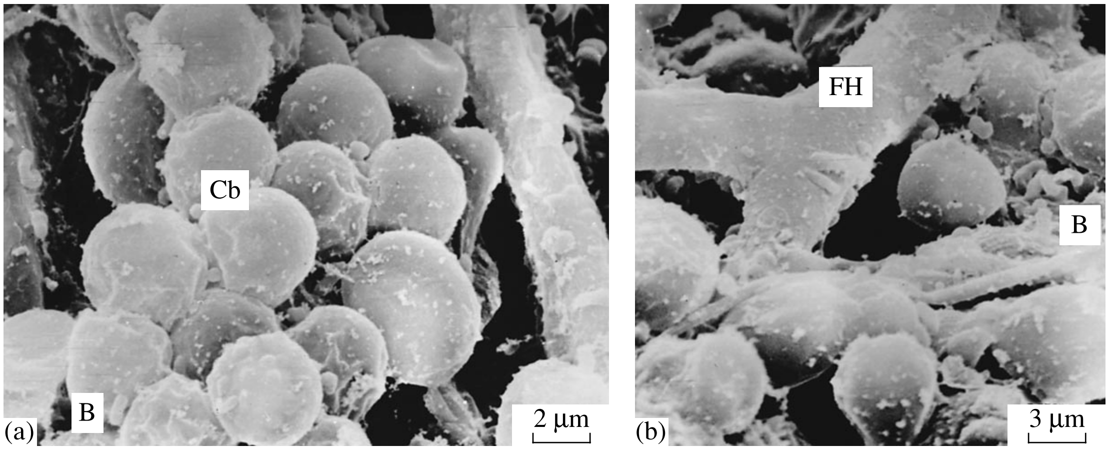

Fig. 6. (a, b) Fragments of the *D. phalaenopsis* aerial root surface. Scanning electron microscopy. B, bacteria; FH, fungal hyphae; Cb, cyanobacteria.

lichens) or with plants (in syncyanoses) during compartmentalization within the thallome or plant tissues [17, 18].

Along with cyanobacteria, bacteria also penetrated the internal layers of the velamen, as shown by the example of *A. papillosa* (Fig. 10). No cyanobacteria were detected within the substrate of *A. papillosa* and *D. moschatum* roots by scanning electron microscopy of root sections. Microscopy of the root surface revealed numerous coccus- and rod-shaped bacterial cells in the slime of a cyanobacterial community (Figs. 3–7).

An insufficient amount of bound nitrogen may be the factor limiting the development of epiphytic orchids. Involvement of various species of cyanobacteria in the formation of the root sheath suggests that fixation of molecular nitrogen is one of their functions in the consortium. In addition, slimy sheaths of cyanobacteria are a favorable econiche for population by bacteria of various species, including nitrogen-fixing ones; in nature, this leads to the formation of various functionally specific consortiums [19].

Potential NFA of microorganisms was measured for aerial roots of *P. amabilis*, covered by the most developed sheaths. The NFA of the root fragments comprised 798.95 nmol ethylene/(h g). This value, calculated per gram native biomass, is the most correct, since other methods of calculation (per unit of chlorophyll, area of the root surface, total protein, or dry biomass weight) do not account for the NFA of the bacterial component of the consortium or the content of chlorophyll in the roots themselves and non-nitrogen-fixing phototrophic microorganisms, as well as the facts that the sheath is a multilayered structure and that microorganisms also occur within the velamen. It is important that we demonstrated for the first time functional activity of microorganisms, namely, nitrogen fixation, in the sheath of the aerial roots of epiphytic orchids. Cyanobacteria may facilitate the energy-consuming process of nitrogen fixation in bacteria, because cyanobacterial slime not only protects heterotrophic microorganisms against drying, but also serves as a nutrient and energy source. Along with substances of carbohydrate nature, the cyanobacterial slime accumulates organic acids, peptides, vitamins, and other compounds [19]. The satellite bacteria are in turn able to remove toxins produced by cyanobacteria and utilize oxygen, which inhibits nitrogen fixation. Bacteria populating the plant root surface and sometimes penetrating the intercellular space of the parenchyma were shown to influence favorably the development and productivity of the colonized plants, because they produce various biologically active substances, including phytohormones [20].

Thus, our study revealed for the first time the presence of cyanobacteria of various morphotypes, as well as other microorganisms, in the rhizoplane and velamen of the roots of epiphytic orchids. Microorganisms differed in their population density on the surface of

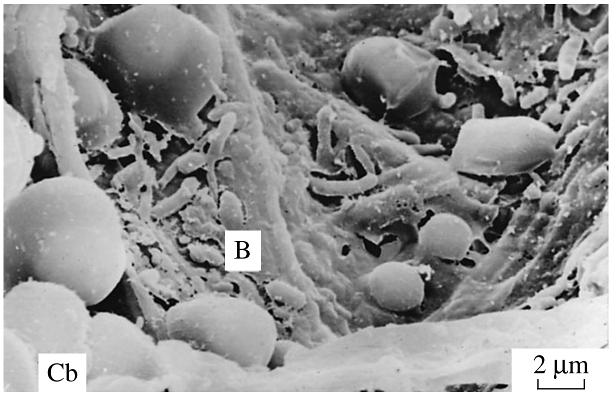

Fig. 7. Fragment of the *D. phalaenopsis* aerial root surface. Scanning electron microscopy. B, bacteria; Cb, cyanobacteria.

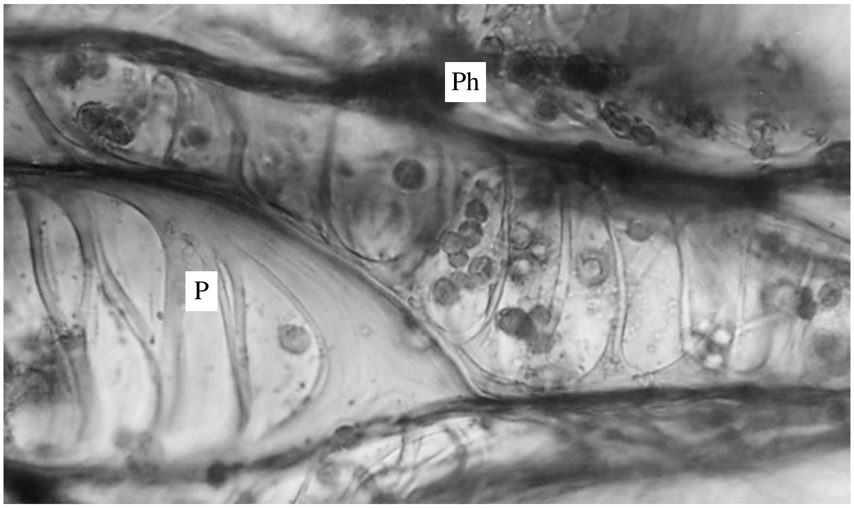

Fig. 8. Longitudinal section of the *D. phalaenopsis* aerial root velamen. Light microscopy. P, velamen perforations; Ph, phototrophic microorganisms; 3300×.

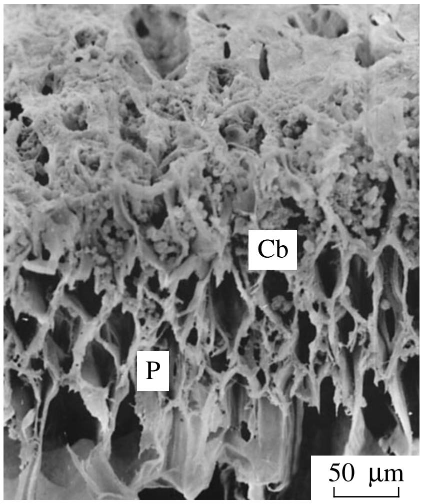

Fig. 9. Fragment of a cross section of the *A. papillosa* aerial root velamen. P, velamen perforations; Cb, cyanobacteria.

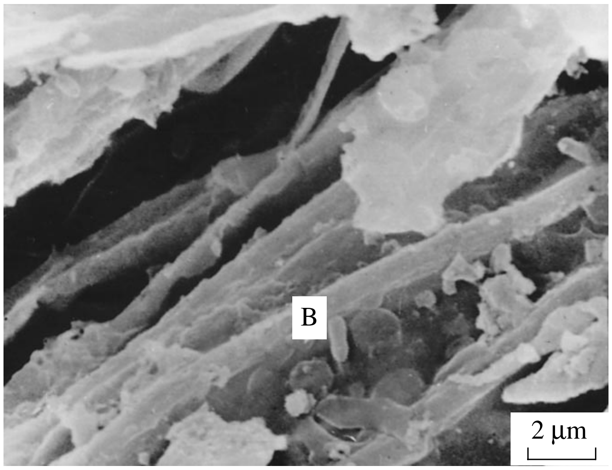

Fig. 10. Fragment of a cross section of the *A. papillosa* velamen (inner side). Scanning electron microscopy. B, bacteria.

aerial and substrate roots of the orchid species examined. The formation of bacterial communities depended on the conditions of plant growth: in climatic conditions similar to those of a moist tropical forest, aerial roots were heavily populated with phototrophic microorganisms. On the surface of aerial roots and in the velamen, the microbial consortium consisted of both photo- and heterotrophic microorganisms; cyanobacteria were the dominant phototrophic component. Microorganisms on the multilayered sheath of *P. amabilis* were shown to actively fix atmospheric nitrogen. This association between the host plant and photo- and heterotrophic microorganisms may be considered an analogue of a nonstructured three-component lichen.

Further studies are required to isolate cyanobacteria and bacteria colonizing orchid roots, to determine their species composition, and to elucidate their role in the formation and stable functioning of the consortium. Understanding of the associative relationships between the orchids and the satellite microorganisms will be helpful for conservation and extension of the collection of orchid species. Attempts to cultivate many of them have been often hitherto unsuccessful because of insufficient knowledge of their peculiar biology.

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