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## Microbiota of the Orchid Rhizoplane

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**Abstract**—Six bacterial strains isolated from the underground roots of the terrestrial orchid *Calanthe vestita* var. *rubro-oculata* were found to belong to the genera *Arthrobacter*, *Bacillus*, *Mycobacterium*, and *Pseudomonas*. Strains isolated from the aerial roots of the epiphytic orchid *Dendrobium moschatum* were classified into the genera *Bacillus*, *Curtobacterium*, *Flavobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, and *Xanthomonas*. The rhizoplane of the terrestrial orchid was also populated by cyanobacteria of the genera *Nostoc* and *Oscillatoria*, whereas that of the epiphytic orchid was populated by one genus, *Nostoc*. In orchids occupying different niches, the spectra of the bacterial genera revealed differed. The microbial complex of the terrestrial orchid rhizoplane differed from that of the surrounding soil.

**Key words:** orchids, rhizoplane, bacteria, cyanobacteria.

Orchids are a very large family of monocotyledonous plants, which includes about 25000 species and 150000 hybrids. Orchids can easily adapt to any habitat, which is why they are now ubiquitous in diverse environments, from equatorial to subantarctic, although the family is primarily tropical [1]. Among orchids, there are epiphytic species, which live on trees and produce aerial roots; terrestrial species with underground roots and bulbs; and mycotrophic species, which lost the capability for photosynthesis. Because of the small amount of nutritive material, tiny orchid seeds need a mycorrhizal fungus to germinate. The dependence on the fungus changes during the course of the orchid ontogenesis [2]. Mature photosynthesizing plants with a large leaf surface may dispense with the fungus, whereas the orchids lacking chlorophyll need this symbiotic fungus throughout their lives [1]. Some problems of the symbiotic association of orchids and fungi remain to be elucidated. For instance, it is unknown why mycorrhizal fungi sometimes kill orchid seedlings [3]. Another problem is the strong dependence of the mycorrhizal fungus on satellite bacteria [4], which are often called helper bacteria [5].

Some soil bacteria, such as pseudomonads, may promote the development of vesicular–arbuscular mycorrhizas [6]. The inoculation of *Stylosanthes guyanensis* plants with a mixture of rhizobial bacteria and *Endogone* fungal spores led to a 6-fold increase in plant biomass and up to a 14-fold increase in its phosphorus

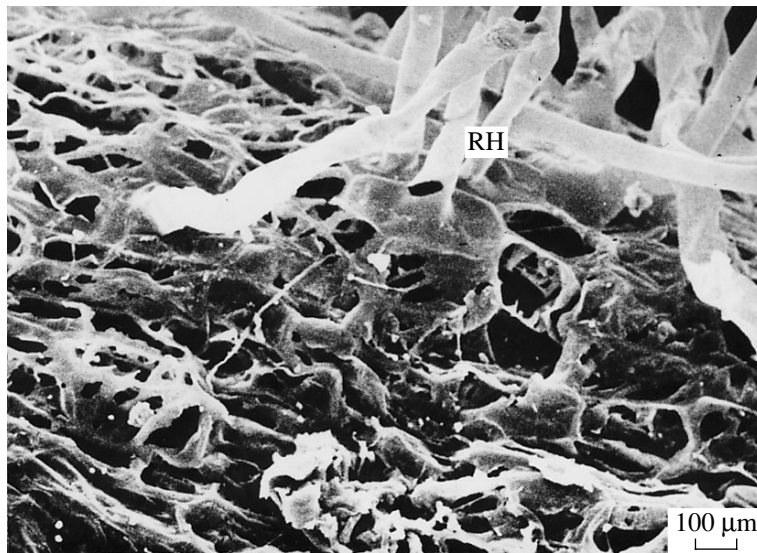
content. The roots of the plants inoculated with this mixture contained, on the average, 34 symbiotic nodules, whereas the roots of the control plants, which were inoculated with only rhizobacteria, contained no nodules [7]. Similar results were obtained by Chanway and Holl [8], who inoculated pine seedlings with a mixture of a mycorrhizal fungus and a plant growth-promoting *Bacillus* strain [8].

As early as in 1922, Knudson showed that the inoculation of orchid seeds with an azotobacter and the root-nodule bacterium *Bacillus radicola* promotes their germination [9]. More recent studies showed that the enhancement of the germination of orchid seeds by the orchid-associated bacteria of the genera *Pseudomonas*, *Bacillus*, *Arthrobacter*, and *Xanthomonas* is mediated by the phytohormone auxin [10, 11]. There is also evidence that the velamen (a spongy tissue covering roots) of orchids is populated by cyanobacteria [1].

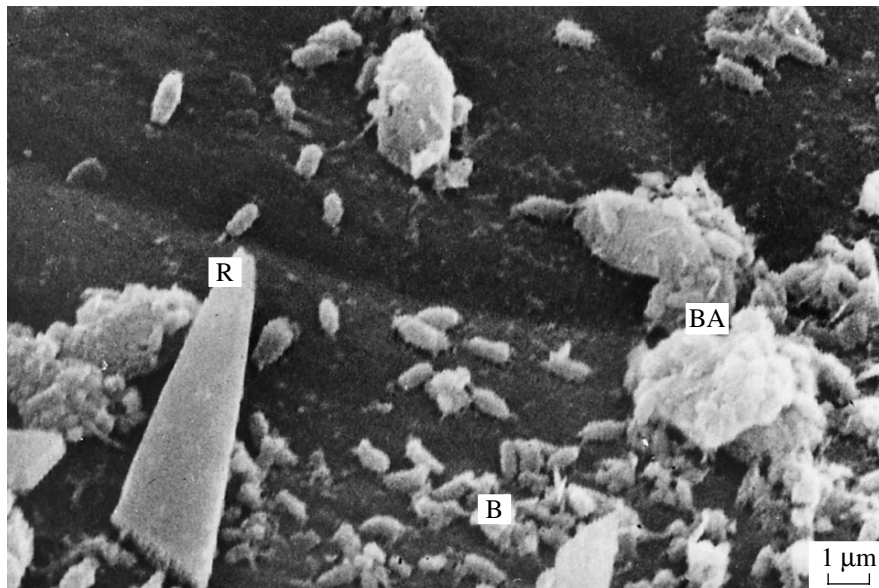
The associations of cyanobacteria and bacteria with mycorrhizal fungi and symbiotic plants are still poorly studied. In particular, little is known about the qualitative and quantitative composition of such associations and about the process of the colonization of the epiphytic orchid roots by microorganisms.

The aim of the present study was to isolate and identify heterotrophic and phototrophic bacteria from the aerial roots of *Dendrobium moschatum* and the underground roots of *Calanthe vestita* var. *rubro-oculata*.

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**Fig. 1.** Scanning electron microscopy of a fragment of the *Calanthe vestita* var. *rubro-oculata* underground root: RH, root hairs.



**Fig. 2.** Scanning electron microscopy of a fragment of the *Dendrobium moschatum* aerial root. B, bacteria; BA, bacterial agglomerates; R, calcium oxalate raphides.

#### MATERIALS AND METHODS

Mature plants of the epiphytic orchid *Dendrobium moschatum* and the terrestrial orchid *Calanthe vestita* var. *rubro-oculata* were grown in the Stock Greenhouse of the Tsitsin Central Botanical Garden in Moscow. *D. moschatum* is an epiphyte growing at the tops of tall deciduous trees under the conditions of bright illumination and extensive air movement. *C. vestita* is a terrestrial deciduous orchid with a root system that is renewed annually.

The *D. moschatum* plants grew on a mixture of fern roots and sphagnum (3 : 1) and the *C. vestita* plants grew on a mixture of leaf litter, turf, black peat, and sand (1 : 1 : 1 : 2).

Experiments were performed with the roots of one-year-old orchids. The roots were collected, together with the growth substrates, using sterile tweezers and spatules and placed in sterile jars. On the same day, the roots, taken in an amount of 1 g, were washed with 100 ml of sterile tap water in a shaker (120 rpm) for 10 min and cut into 5-mm-long fragments with a sterile razor. Four

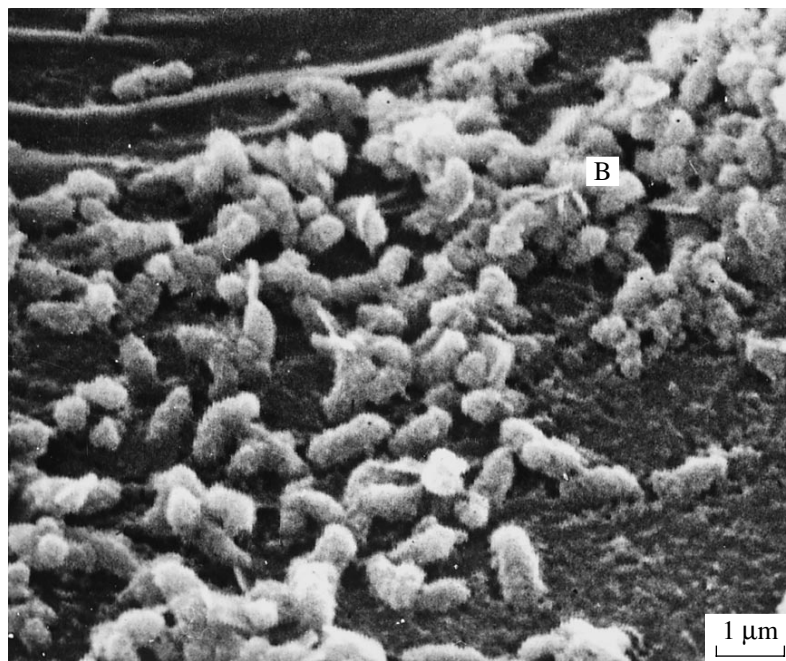


Fig. 3. Scanning electron microscopy of a fragment of the *D. moschatum* aerial root. B, bacteria.

to five randomly taken root fragments were placed in a small serum vial with 6 ml of Czapek medium containing (g/l) glucose, 20;  $\text{NaNO}_3$ , 2.0;  $\text{K}_2\text{HPO}_4$ , 1.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5;  $\text{KCl}$ , 0.5; and  $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ , 0.1 in distilled water. The vials were plugged with cotton stoppers and incubated at  $28^\circ\text{C}$  in the dark on a shaker for 4 weeks. At one-week intervals, the contents of the vials were plated on the aforementioned medium solidified with agar. To prevent the growth of fungi, the agar medium was supplemented with  $50 \mu\text{g/ml}$  nystatin. Pure cultures were obtained from individual colonies by the Koch method and maintained on half-strength nutrient agar.

To isolate cyanobacteria, 10–15 root fragments were placed in 50-ml flasks with 30 ml of  $\text{BG}_N\text{-11}$  medium either with or without nitrogen sources [12] and incubated at room temperature and an illumination of 700 lx for 2 months.

For the sake of the comparison of the bacterial complexes in the *Calanthe* rhizoplane and the surrounding soil, the latter was incubated under the same conditions as the root fragments, and its bacterial population was evaluated by the same methods as that of the roots. The soil was incubated in Czapek and  $\text{BG}_N\text{-11}$  media in amounts of 0.3 g/6 ml and 0.5 g/30 ml, respectively.

Bacteria were identified by the routine methods [13, 14] based on their cultural, morphological, physiological, and biochemical characteristics: the presence of oxidases and catalase; the ability to denitrify nitrates, to utilize glucose and lactose, and to grow at pH 4.5; the response to a test for the oxidative utilization of glucose; amylolytic and proteolytic activities; acid resis-

tance (in the case of mycobacteria); and spore formation (in the case of bacilli). *Nocardia* were identified using Gauze medium and oat agar (to test the formation of micelles). All experiments were performed in triplicate. Identification at a species level was not carried out.

For scanning electron-microscopic studies, the roots were fixed for 30 min with a 2% solution of glutaraldehyde in the Millonig buffer [15] and dehydrated in ethanol solutions of increasing concentration. After the final dehydration in absolute ethanol and then overnight in 100% acetone, the roots were freeze-dried using an HCP-2 device (Hitachi, Japan), coated with Au-Pd in an IB-3 device (Hitachi), and examined with an 1830I scanning electron microscope (Amray, United States).

## RESULTS

The scanning electron-microscopic studies of the orchid rhizoplane showed that the continually growing velamen (a spongy coating tissue) of the roots (Fig. 1) is a good econiche for microorganisms (Fig. 2–6). The surface of the aerial roots of *D. moschatum* exhibited the presence of numerous cells of different morphology, which were located around the needle-shaped crystals of calcium oxalate exuded by the roots (the so-called raphides) (Fig. 2). Along with individual bacterial cells about  $1 \mu\text{m}$  in size (Fig. 3), there were cell agglomerates submerged in an intercellular matrix (Figs. 2 and 6) and the aggregates of spherical cells close to cyanobacteria in size (Figs. 4 and 6). On the underground roots of the *C. vestita* var. *rubro-oculata*, we also detected fungal hyphae (Fig. 5).

Among the six bacterial isolates obtained from these roots, three were gram-positive and three were gram-negative. The colonies of these isolates were primarily white; however, the colonies of one isolate were cream-orange, and the white colonies of another isolate had yellow strands on their edges. The consistency of almost all the colonies was mucoid, except that the colonies of one isolate were pasty.

Among the eight isolates obtained from the aerial roots of *D. moschatum*, three were gram-positive and five were gram-negative. Only two isolates produced white colonies, whereas the colonies of the other isolates were beige, brightly yellow, orange, and red. The colony consistency was primarily pasty, except that the colonies of one isolate were mucoid, and the colonies of another isolate were dry and mycelium-like.

Three bacterial isolates obtained from the underground roots of *C. vestita* var. *rubro-oculata* were found to belong to the genera *Arthrobacter*, *Bacillus*, and *Mycobacterium*, whereas the other three isolates belonged to the genus *Pseudomonas*. Six strains isolated from the aerial roots of *D. moschatum* were classified into the genera *Bacillus*, *Curtobacterium*, *Flavobacterium*, *Nocardia*, *Pseudomonas*, and *Rhodococcus*, and two strains were found to belong to the genus *Xanthomonas*. The bacteria common to the rhizoplane of the terrestrial orchid were not detected in the rhizosphere soil.

As for cyanobacteria, they were not detected in the soil samples even if the latter were incubated for two months in nutrient media with or without nitrogen sources. At the same time, the underground roots of *C. vestita* var. *rubro-oculata* gave rise to numerous colonies of the cyanobacterium *Oscillatoria* and to a few colonies of the cyanobacterium *Nostoc* on the nitrogen-containing agar. The latter genus could also be detected on the nitrogen-free agar. The microbial complex of the *D. moschatum* roots included only one cyanobacterial genus, *Nostoc*.

Microbiological analysis using agar media without nystatin confirmed the presence of fungi in the orchid rhizoplane: the underground roots of *C. vestita* var. *rubro-oculata* exhibited the presence of two fungal morphotypes and the aerial roots of *D. moschatum* exhibited one fungal morphotype.

## DISCUSSION

The results obtained in this study show that the orchid rhizoplane is well populated by bacteria and cyanobacteria due to the presence of the spongy velamen, which passes gases (thus creating a microoxic environment) and accumulates substances that migrate to the roots from the surrounding soil. The aerial roots of the epiphytic orchid and the underground roots of the terrestrial orchid were populated by different bacterial genera. Furthermore, the microbial complex of the rhizoplane of the terrestrial orchid differed from the



Fig. 4. Scanning electron microscopy of a fragment of the *D. moschatum* aerial root. B, bacteria; CB, cyanobacteria.

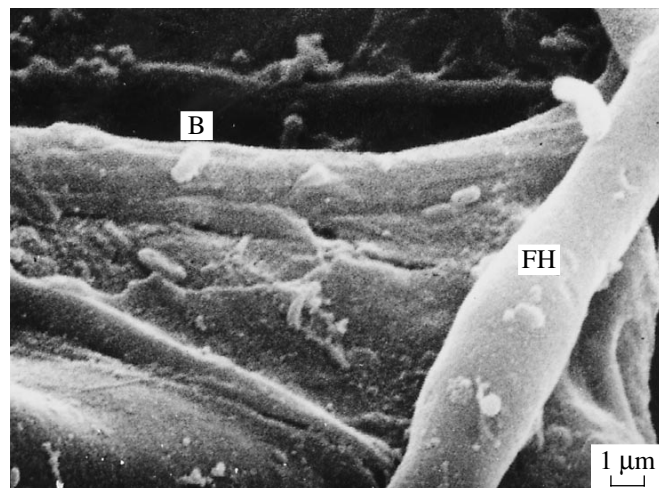


Fig. 5. Scanning electron microscopy of a fragment of the *C. vestita* var. *rubro-oculata* underground root. B, bacteria; FH, fungal hyphae.

microbial complex of the surrounding soil. Three of the six isolates obtained from the underground orchid roots were found to belong to the genus *Pseudomonas*. This finding agrees with the data available in the literature that this genus is the most frequent plant endosymbiont and the common inhabitant of the rhizoplane and rhizosphere [16, 17]. According to the data of Wilkinson

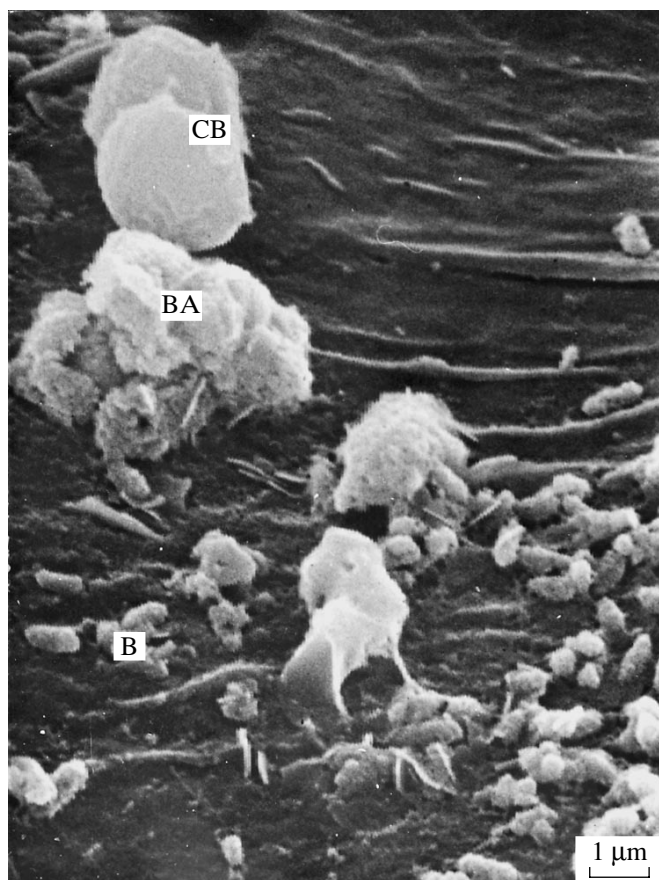


Fig. 6. Scanning electron microscopy of a fragment of the *D. moschatum* aerial root. B, bacteria; BA, bacterial agglomerates; CB, cyanobacteria.

*et al.* [10, 11], bacteria of the genera *Pseudomonas*, *Xanthomonas*, *Bacillus*, and *Arthrobacter* are the endosymbionts of West Australian orchids, inhabiting their underground roots. The proportion between these bacterial genera varied depending on the orchid species, season, the age of roots, and the degree of their colonization with a mycorrhizal fungus [10].

All rhizoplane bacteria, except for *Nocardia*, produce colonies with a developed intercellular matrix, which not only fixes bacterial cells on the root surface but also creates a beneficial microenvironment for them, accumulating nutritive and regulatory substances and ions [18]. In addition, this matrix protects the attached bacterial cells from desiccation, radiation, and the action of other detrimental factors and performs an ecological function, structurizing natural biocenoses [18].

Like the pigments of photosynthesizing bacteria, the pigments of the bacteria isolated from the aerial roots of *Dendrobium* may protect these bacteria from the harmful action of ultraviolet radiation, as this epiphytic orchid lives under direct sunlight.

Many isolated genera are known to include nitrogen fixers. Cyanobacteria of the genus *Nostoc*, which are

associated with both of the orchids studied here, are diazotrophs producing heterocysts in nitrogen-deficient media. The problem of the taxonomic assignment of orchid-associated cyanobacteria has not yet been sufficiently studied, although it has been recognized that the cyanobionts of plant syncyanoses belong to the order *Nostocales*, primarily to the genera *Nostoc* and *Anabaena* [19].

The fact that cyanobacteria of the genera *Nostoc* and *Oscillatoria* were isolated from the underground roots of *C. vestita* var. *rubro-oculata* confirms that these photosynthesizing bacteria are able to grow not only photomixotrophically and photoheterotrophically but also on organic substrates in the dark [20].

Thus, we showed the presence of specific microbiota on the surface of the orchid roots. The research along this line should be continued in order to elucidate the species fidelity of microorganisms to the particular type of orchid roots and the physiological and biochemical properties of the isolated microbial symbionts, primarily with reference to nitrogen fixation and the synthesis of phytohormones and other signal substances. This should greatly contribute to understanding the formation, function, and maintenance of the host plant–mycorrhizal fungus–bacterium symbioses.

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