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EXPERIMENTAL ARTICLES

Microbial Complexes Occurring on the Apogeotropic Roots and in the Rhizosphere of Cycad Plants

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Abstract—The microbial complexes of soil, the rhizosphere, and the rhizoplane of the apogeotropic (coralloid) roots of cycad plants were comparatively studied. The aseptically prepared homogenates of the surface-sterilized coralloid roots did not contain bacterial microsymbiont, indicating that in the root tissues the symbiosis is a two-component one (plant–cyanobacteria). At the same time, associated bacteria belonging to different taxonomic groups were detected in increasing amounts in the cycad rhizoplane, rhizosphere, and the surrounding soil. The bacterial communities found in the cycad rhizoplane and the surrounding soil were dominated by bacteria from the genus *Bacillus.* The saprotrophic bacteria and fungi colonizing the cycad rhizosphere and rhizoplane were dominated by microorganisms capable of degrading the plant cell walls. The local degradation of the cell wall was actually observed on the micrographs of the thin sections of cycad roots in the form of channels through which symbiotic cyanobacterial filaments can penetrate into the cortical parenchyma.

Key words: cycads, apogeotropic roots, microbial complexes, rhizoplane, rhizosphere.

Cycadales is the only order of the gymnospermous class *Cycadopsida* that contains living species, whereas the three other orders of this class are known only from fossils. Cycads are woody plants of the family *Cycadaceae*, which consists of nine to eleven genera and amounts to 150 tropical and subtropical species. Among gymnosperms, *Cycadales* is the second-most-abundant order (after *Coniferales*) in the number of living species [1].

The root system of cycad plants is characterized by the development of specific, dichotomous, apogeotropic, aerial roots (also called coralloid roots), which occur, as small root clusters, on the soil surface or in the air at heights of up to 10 cm [2]. Coralloid roots develop from secondary lateral roots and contain microbial associations dominated by nitrogen-fixing cyanobacteria [3, 4]. Little information is presently available as to the abundance, species composition, and the specific role of microsymbionts in the development and life of symbiotic associations with cycad plants [4, 5].

In recent years, bacteria colonizing the rhizoplane (i.e., the external surface of roots together with the outer cell layer of the root epidermis (periderm)) have been attributed to associated bacteria, which are believed to be important in the formation of symbiosis and the stable coexistence of symbiotic partners [6]. There is evidence that the exometabolites produced by the associated bacteria beneficially influence both the phytosymbiont and the dominant microsymbiont and markedly extend the ecological conditions under which their symbiosis is stable [6–8]. The primary isolates of cyanobionts recovered from the coralloid roots of various cycad species always contain heterotrophic bacteria [9, 10], whose abundance and taxonomic composition are as yet to be appropriately studied.

The aim of this work was to study the taxonomic composition of heterotrophic microbial complexes occurring on the apogeotropic roots and in the rhizosphere of cycad plants.

MATERIALS AND METHODS

Experiments were carried out with fragments of the apogeotropic roots of the *Cycas circinalis* L., *Ceratozamia mexicana* Brough., *Encephalartos villosus* Lehm., and *E. hindelbrantii* Broun et Bouche cycad plants grown in a subtropical greenhouse of the Tsitsin Central Botanical Garden. The two *E. villosus* plants were 30 and 50 years old; the *C. circinalis* and *C. mexicana* plants were 50 years old; and the *E. hindelbrantii* plant was 200 years old. Samples of the apogeotropic roots of the plants and the surrounding soil were collected in June 2001.

Investigated were microbial complexes in the soil in which the cycad plants were grown; the aerial cycad rhizosphere; the cycad rhizoplane; and the aseptically prepared homogenates of surface-sterilized cycad roots. Samples of the apogeotropic roots represented standard fragments (5 to 7 mm long) of their apical ends. The rhizosphere and rhizoplane samples were prepared as follows: The apogeotropic root fragments were placed in 20 ml of sterile tap water and shaken at

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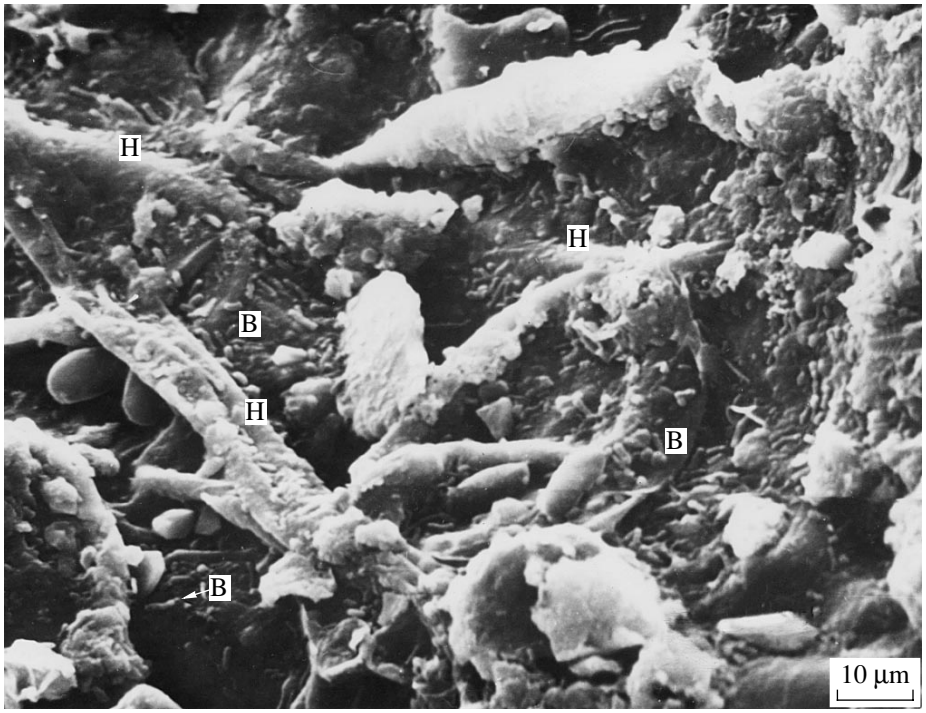

Fig. 1. A scanning electron microscopic image of the surface of the apogeotropic root of *E. villosus*. H, hyphae; B, bacteria.

180 rpm for 3 min. The liquid phase after this procedure was considered a sample of the cycad rhizosphere. The roots were collected, transferred to the next 20-ml portion of sterile tap water, and sonicated for 2 min using a UZDN-1 ultrasonic generator (22 kHz, 0.44 A) in order to desorb microorganisms attached to the root surface. The liquid phase thus prepared was considered a sample of the cycad rhizoplane. After this step, the roots were surface-sterilized in 0.1% mercuric chloride for 15 min, washed three times with sterile water,

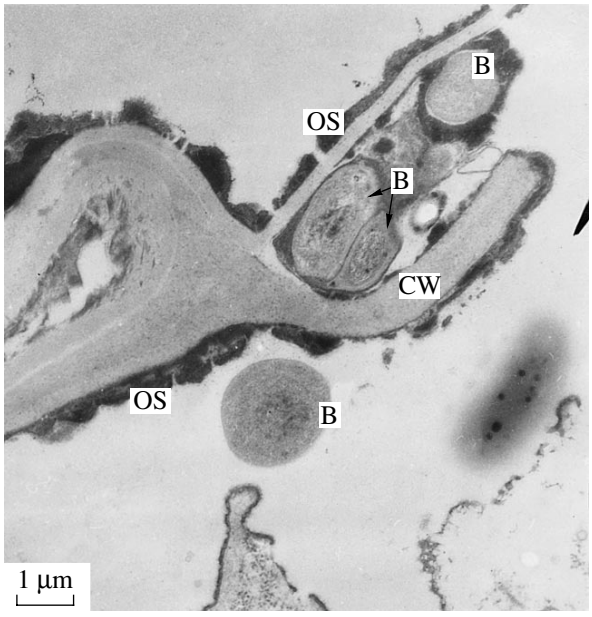

Fig. 2. Bacterial cells (B) in a periderm cell wall (CW) wrinkle in the apical zone of the apogeotropic root of *E. villosus*. OS, osmophilic substance.

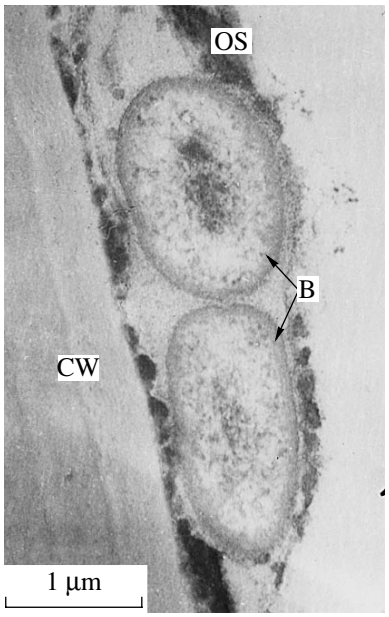

Fig. 3. Bacterial cells (B) immobilized on the plant cell surface in the apical zone of the apogeotropic root of *E. villosus*. OS, osmophilic substance.

The population density of (I) bacteria and (II) actinomycetes in the root zone of cycad plants as determined by the plating method and expressed in thousand CFU/g apogeotropic roots

Cycad plant	Soil		Rhizosphere		Rhizoplane	
	I	II	I	II	I	II
C. circinalis (30-year-old)	28300	3200	19200	1400	5900	72
C. mexicana (30-year-old)	11000	2600	7600	970	560	55
E. villosus (30-year-old)	–	–	1000	200	1400	140
E. villosus (50-year-old)	320	30	156	19	140	11
E. hindelbrantii (200-year-old)	42	3.4	36200	61400	15800	7000

Note: The dashes indicate that no measurements were carried out.

placed in 30% hydrogen peroxide for 20 min, and again washed three times with sterile tap water. Then the roots were thoroughly homogenized, and the homogenate was suspended in 20 ml of sterile tap water. The suspension was referred to as a root homogenate.

Bacteria and streptomycetes were counted using a modified glucose–peptone–yeast extract medium [11]. To inhibit the growth of fungi, the medium was supplemented with 100 mg/l nystatin. Appropriately diluted samples were plated, in five replicates, onto the agar medium, and the plates were incubated at 20°C for 2–3 weeks. The total abundance of prokaryotes was expressed in colony-forming units (CFU) per gram sample. The colonies produced by actinomycetes and bacteria of different taxonomic groups were differentiated according to their morphology, and each colonial morphotype was enumerated separately. Three to five representatives of each colonial morphotype were isolated in pure culture. The isolates were identified on the basis of their morphological, cultural, and chemotaxonomic characteristics using the identification criteria of Bergey's Manual [12]. The streptomycete isolates were identified to the level of species, as recommended by Gauze *et al.* [13]. The morphocultural characteristics of these isolates were determined after 1, 2, and 3 weeks of incubation.

The occurrence rate of bacteria was defined as the ratio of the number of samples in which a given bacterial taxonomic group was detected to the total number of samples examined.

The total number of soil fungi was determined using a Czapek medium with 2% sucrose and 4 ml/l lactic acid (to suppress the growth of bacteria). Soil fungi were identified according to the instructions of Domsch *et al.* [14].

Specimens for electron transmission and scanning microscopy were prepared as described earlier [15].

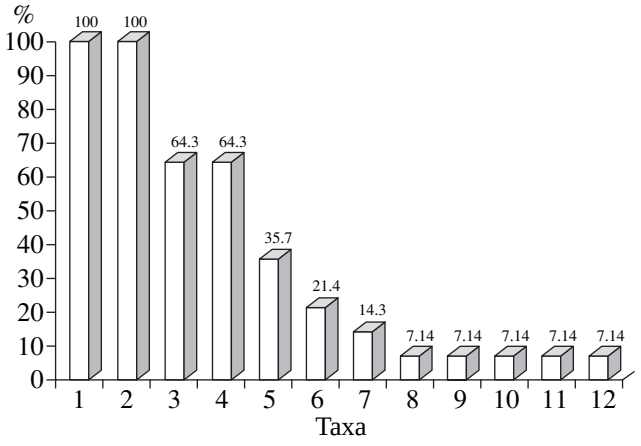

Fig. 4. The occurrence frequency rates (expressed as a percent) of various bacteria in the root zone (rhizosphere, rhizoplane, and surrounding soil) of the cycad plants. Bacterial taxa: (1) *Bacillus*, (2) *Streptomyces*, (3) *Arthrobacter*, (4) *Myxobacterales*, (5) *Rhodococcus*, (6) *Cytophaga*, (7) *Cellulomonas*, (8) *Aquaspirillum*, (9) *Azotobacter*, (10) pigmented coryneform bacteria, (11) *Flavobacterium*, and (12) small coryneform bacteria.

RESULTS AND DISCUSSION

The examination of the apogeotropic cycad roots by scanning electron microscopy showed the presence on the root surface of a large number of bacterial cells of different morphotypes and mycelial hyphae, often submerged in a slimy matrix (Fig. 1). The scanning electron microscopy of the coralloid root sections revealed the presence of bacteria in the intercellular space of periderm and in the outer cell layer of cortical parenchyma. The analysis of thin sections of the apical zone of the apogeotropic roots showed the presence of bacterial cells, often localized in a homogeneous electron-dense material, in the cell wall folds of dead periderm cells (Fig. 2). Some bacterial cells were in contact with the plant cell wall or even partially penetrated it (Fig. 3). Symbiotic cyanobacterial cells were localized, in the form of a ring 1 to 3 mm in thickness, in the cell layer of the cortical parenchyma of the coralloid roots.

At the same time, scanning and transmission electron microscopy did not reveal bacterial cells in the apogeotropic root fragments. Nor could we detect bacteria in the root homogenates of any of the cycads under study. These data suggest that nitrogen-fixing cyano-

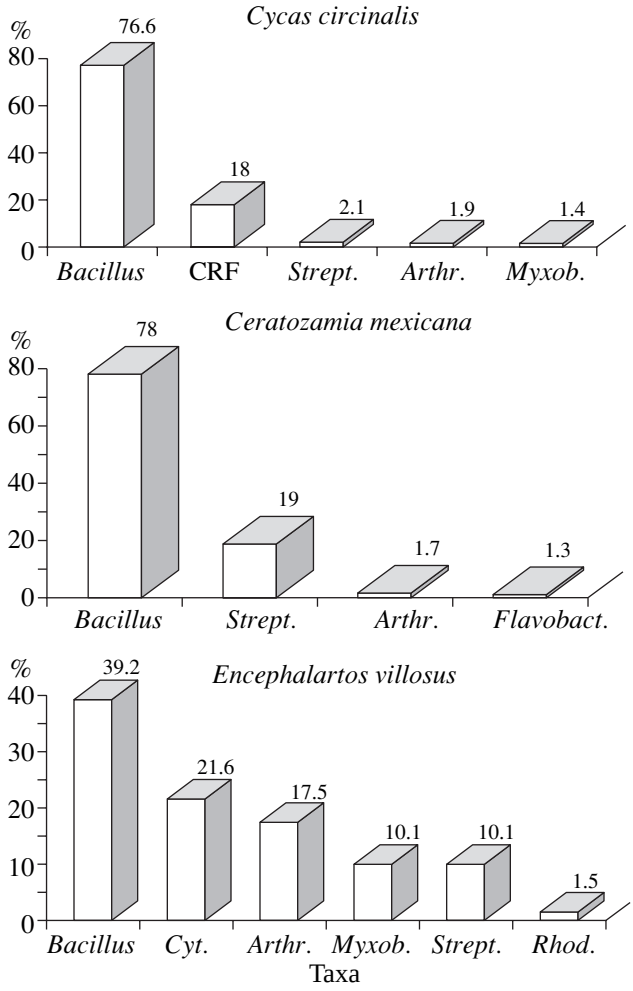

Fig. 5. The relative abundances (expressed as a percent) of various bacteria in the rhizoplane of the 50-year-old *E. villosus*, *C. mexicana*, and *C. circinalis* plants. CRF is coryneform bacteria, *Cyt.* is *Cytophage*, *Art.* is *Arthrobacter*, *Myxob.* is *Myxobacterales*, *Strept.* is *Streptomyces*, *Rhod.* is *Rhodococcus*, and *Flavobact.* is *Flavobacterium.*

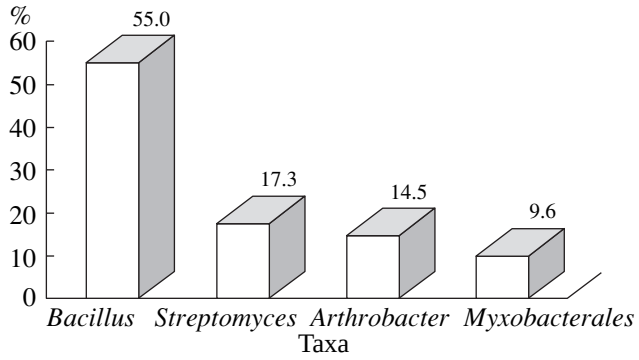

Fig. 6. The relative abundances (expressed as a percent) of various bacteria in the greenhouse soil in which the cycad plants were grown.

bacteria are the only microsymbiont of the cycad root tissue.

The microbial population in the root zone of the cycad plants decreased in the following order: surrounding soil (11–28 million CFU/g) > rhizosphere > rhizoplane (0.1–5 million CFU/g) (table). The decline in the actinomycete population was even steeper (from 3 million CFU/g in the surrounding soil to 0.01 million CFU/g in the rhizoplane). A similar distribution pattern was observed for micromycetes, whose population in the surrounding soil, rhizosphere, and rhizoplane of the *C. mexicana* plant comprised 90, 8.5, and 4.25 thousand CFU/g, respectively.

It should be noted that the distribution pattern of microorganisms in the root zone of woody and herbaceous plants growing under natural conditions usually differs from the distribution pattern described above. Namely, the microbial population (determined by the method of plating) is higher in the plant rhizosphere and rhizoplane than in the surrounding soil (the so-called rhizosphere effect). Cycad plants are distinguished by the presence of apogeotropic (or aerial) roots. Therefore, the rhizosphere of cycad plants is aerial and, hence, its lower microbial population seems to be justified. On the other hand, the microbial population in the rhizosphere of the 200-year-old *E. hindelbrantii* plant proved to be several orders of magnitude denser than in the surrounding soil. This fact can be explained by the processes associated with the dying out and degradation of cycad roots.

The saprotrophic bacteria isolated from the root zone of the cycad plants comprised 12 taxa. The genera *Bacillus* and *Streptomyces*, whose representatives were found in the majority of the samples studied, were the most frequent taxa. Myxobacteria and arthrobacter were the second most frequently isolated taxa. The representatives of other taxa (including coryneform bacteria from the genera *Cellulomonas* and *Rhodococcus* and gram-negative bacteria from the genera *Cytophaga*, *Flavobacterium*, *Aquaspirillum*, and *Azotobacter*) were the third most frequently identified bacteria (they were isolated from 7 to 35% of samples taken for analysis) (Fig. 4).

The bacterial communities of the aerial rhizoplane of the cycad plants and the surrounding soil were dominated by bacteria of the genus *Bacillus* (Figs. 5, 6). The relative abundances of gliding bacteria (myxobacteria and cytophages), streptomycetes, and coryneform bacteria amounted to 18–20% (Fig. 5). The bacterial complex of the rhizosphere of the 200-year-old cycad plant was the least diverse, containing only two dominant genera (*Bacillus* and *Streptomyces*), while the occurrence frequency of myxobacteria was as low as about 4%. In spite of this, the microbial population density in the rhizoplane of coralloid roots was several orders of magnitude higher than in the surrounding soil.

According to earlier observations [16], the coralloid root tissues of cycad plants are able to synthesize and accumulate up to 11 phenolic compounds of different

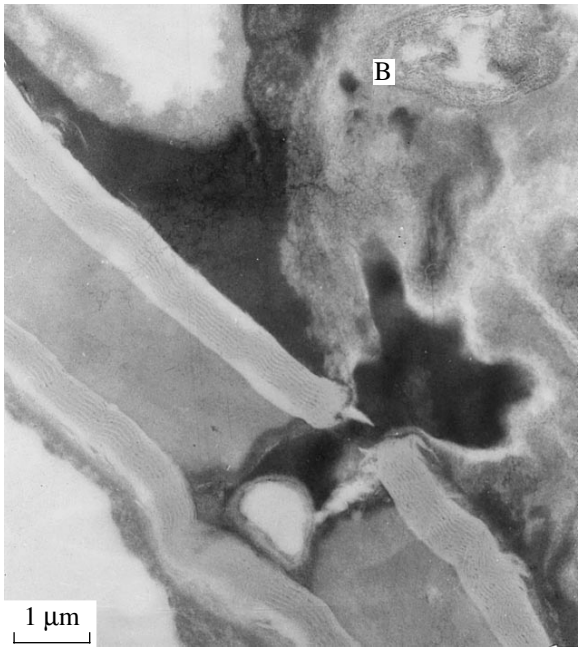

Fig. 7. A channel in the periderm cell wall of the apogeotropic root of *C. circinalis*, through which bacterial cells can penetrate into the cortical parenchyma.

groups. In order of increasing amounts of chemically detected phenolic compounds and flavanes histochemically detected in their coralloid roots, cycad plants ranked as *C. mexicana* > *E. villosus* > *C. circinalis* [17]. It is known that phenolic compounds play an important protective role, preventing the propagation of phytopathogenic microorganisms in infected plants [18]. It is likely that the taxonomic structure and the population density of microbial complexes in the rhizosphere, rhizoplane, and the surrounding soil of particular cycad plants is determined, on the one hand, by the range and the amount of phenolic compounds synthesized in the coralloid roots and, on the other hand, by the resistance of plant-associated microorganisms to these compounds.

The bacterial complex of soil in the greenhouse where the cycad plants were grown was dominated by the same bacterial genera as the bacterial complex of the aerial cycad rhizosphere. The occurrence rate of the typical pedobiont *Arthrobacter* was 12–18% (Fig. 6). Streptomycetes were represented by the species *St. roseus, St. clavuligeris, St. bikiniensis, St. venesuele, St. chrysomallus, St. viridobrunneus,* and *St. bacillaris.*

The predominance of the genera *Bacillus, Streptomyces,* and *Arthrobacter* is typical of most arid, cryoarid, and many mesomorphic soils or soil horizons in which bacteria have long been in a dormant state [19]. Bearing in mind that the cycad plants studied in the present work were grown in an artificially created soil and that the cycad rhizosphere is aerial, we suggested that the taxonomic structure of bacterial complexes in the root zone of the cycad plants must be nonstandard and dominated by bacteria resistant to unfavorable growth conditions.

In nature, syncyanosis is formed de novo in each cycad plant. The coralloid roots of a cycad plant can be colonized by different cyanobacteria but may have no intratissue cyanobacteria [10, 20]. This suggests that the coralloid root tissue is not colonized by the cyanobiont through the system of intercellular gaps. Young apogeotropic roots are colonized by cyanobacteria from the surrounding medium through the outer layers of dead and live root tissue cells, which requires that the plant cell wall be degraded.

Some authors suggested that satellite bacteria localized in the periderm and the outer layers of the cortical parenchyma may play a role in the formation of channels for symbiotic cyanobacteria to penetrate into the root tissue [1, 10, 20]. We succeeded in isolating 150 strains of gram-positive and gram-negative bacteria from the rhizoplane, aerial rhizosphere, and the surrounding soil of the cycad plants. Twenty-six strains of this collection, representing 8 different taxa, were tested for the presence of enzymes capable of degrading the plant cell walls (polygalacturonase and pectin methylesterase). Polygalacturonase was detected in most bacteria of the order *Myxobacterales*; pectin methylesterase was detected in coryneform bacteria from the genera *Arthrobacter, Rhodococcus,* and *Cellulomonas* and in the *Aquaspirillum* bacteria; both enzymes were detected only in four strains of the genera *Arthrobacter* and *Bacillus* (unpublished data of Dol'nikova and Lobakova).

Thus, many saprotrophic bacteria colonizing the root surface of cycad plants (their population density may reach several million cells/g) possess pectinolytic activity and, hence, are able to degrade the primary parenchyma cell walls, thereby facilitating the penetration of cyanobacteria into the root tissue of the infected plant.

The micromycete complex of the cycad plants was found to be dominated by imperfect saprotrophic fungi from the genera *Trichoderma* (*T. harzianum, T. koningii, T. polysporum,* and *T. aureoviride*), *Fusarium, Verticillium,* and *Acremonium.* In addition, we isolated the fungus *Stachybotris parvispora* from the rhizosphere and the rhizoplane of the 30- and 50-year-old *E. villosus* plants and the fungi *Phialophora atrovirens* and *Scopulariopsis brumptii* from the apical fragments of the coralloid roots of *C. circinalis.* All of the biotopes studied were dominated by cellulolytic fungi.

To conclude, the comparative study of the microbial complexes of the aerial rhizosphere, rhizoplane, and the surrounding soil of the cycad plants showed that the aseptically prepared homogenates of the surface-sterilized coralloid roots did not contain bacteria, indicating that bacteria are absent in the root tissues. At the same time, plant-associated bacteria belonging to different taxonomic groups were detected (in increasing

amounts) in the cycad rhizoplane, rhizosphere, and the surrounding soil. The saprotrophic bacteria and fungi colonizing the cycad rhizosphere and rhizoplane were dominated by microorganisms capable of degrading the plant cell walls. The local degradation of the cell wall was actually observed on the micrographs of the thin sections of cycad roots in the form of channels (Fig. 7), through which symbiotic cyanobacterial filaments can penetrate into the cortical parenchyma.

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