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EXPERIMENTAL ARTICLES

Heterocysts with Reduced Cell Walls in Populations of Cycad Cyanobionts

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Abstract—The ultrastructure of the cyanobionts of the greenhouse-grown cycads *Cycas circinalis*, *Ceratozamia mexicana*, and *Encephalartos villosus* was studied. In addition to heterocysts with the typical ultrastructure, the cyanobiont microcolonies also contained altered heterocysts with reduced cell walls, which might dominate in all regions of the coralloid roots. The altered heterocysts represented a protoplast enclosed in a heterocyst-specific envelope with additional layers. Some heterocysts contained an additional reticular protoplast-enclosing sheath below the heterocyst-specific envelope, whereas the other heterocysts contained an additional electron-opaque outer layer. The substance of the inner sheath of the former heterocysts resembled the polysaccharides of mucilage, which fills the intercellular space, whereas the electron-opaque outer layer of the latter heterocysts probably had a protein nature. The substances that constitute the sheath and the outer layer are likely to be synthesized intracellularly and then released with the aid of membrane-bounded vesicles or by ruptures in the cytoplasmic membrane.

Key words: symbiosis, cycads, cyanobionts, heterocysts, ultrastructure, protoplasts, mucilage.

Nitrogen-fixing cyanobacteria (predominantly those of the genus *Nostoc*) can symbiose with higher plants, namely bryophytes, ferns, and gymno- and angiospermous plants. The primary role of a symbiotic cyanobacterium is to fix atmospheric nitrogen and to provide its plant symbiont with bound nitrogen. Molecular nitrogen is fixed by specialized cyanobacterial cells (heterocysts), whose number considerably increases when cyanobacteria pass to a symbiotic state [1]. Cyanobacterial heterocysts differ from vegetative cells in some specific morphological and ultrastructural characteristics corresponding to their specialization [2]. The heterocysts of the cyanobionts of higher plants, namely cycad plants can be heterogeneous in ultrastructure [3–5]. In particular, the heterocysts occurring in the senescent symbiotic organs (apogeotropic roots, called also coralloid roots or coralloids) of cycad plants have a considerably degraded ultrastructure [3]. The heterocysts of the cyanobacterium *Nostoc* in symbiotic associations with the flowering plant *Gunnera kaalensis* were found to have an irregular shape and a highly osmiophilic cytoplasm, because of which

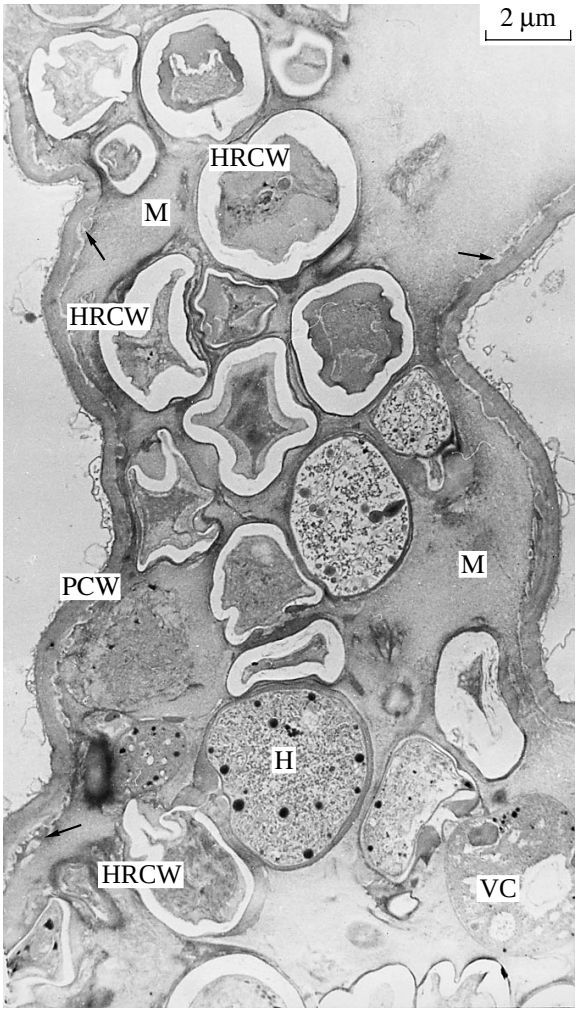

Fig. 1. A cyanobiont microcolony in the intercellular space of the cyanobacterial zone of cortical parenchyma near the coralloid root apex of *E. villosus*. VC, vegetative cell; H, heterocyst; HRCW, heterocyst with the reduced cell wall; M, mucilaginous intercellular matrix; PCW, plant cell wall. The arrows show the interface between the mucilaginous intercellular matrix and the plant cell wall.

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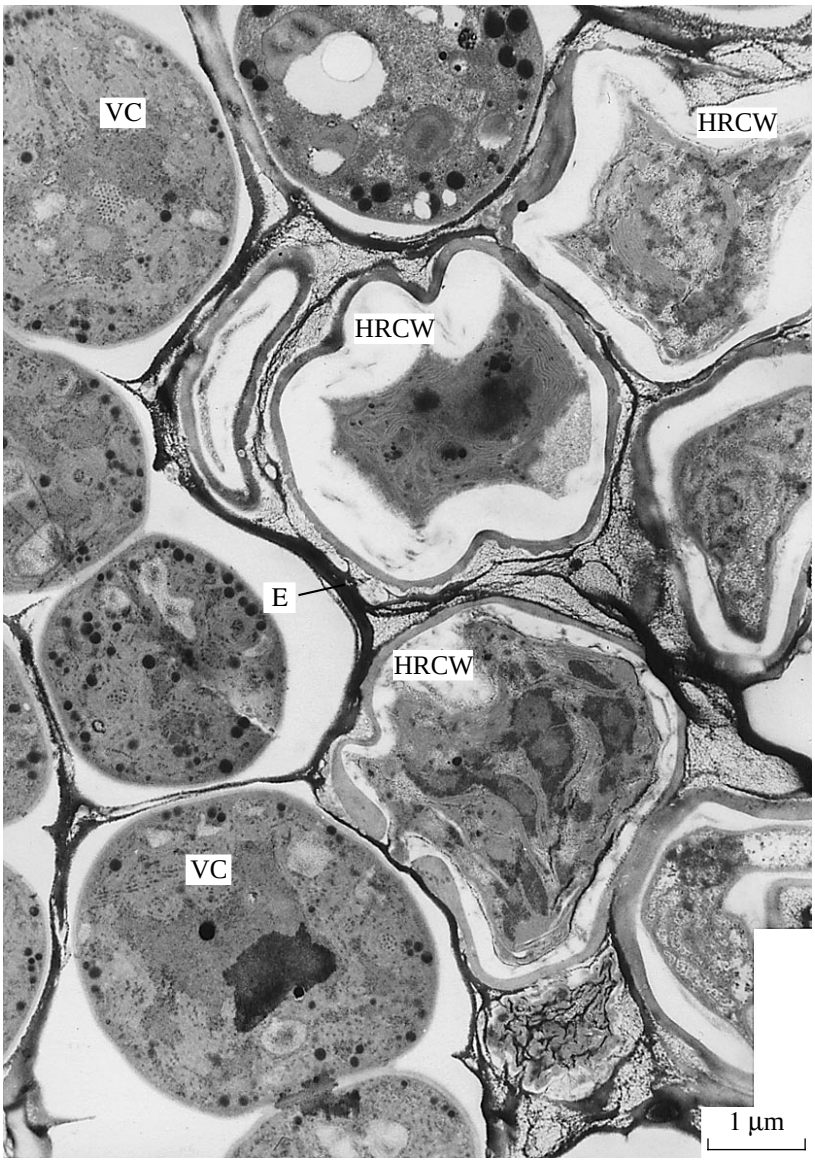

Fig. 2. A cyanobiont microcolony in the intercellular space of the cyanobacterial zone of cortical parenchyma near the coralloid root apex of *C. circinalis.* E, electron-opaque envelope. The other designations are as in the legend to Fig. 1.

they were designated as degenerated heterocysts [6]. Such heterocysts were numerous not only in old but also in young symbiotic tissues. The heterocysts of *Nostoc punctiforme* associated with the hornwort *Anthoceros punctatus* also showed an altered morphology: they were large and irregularly shaped [7]. With respect to the cyanobionts of cycad plants, the altered ultrastructure of heterocysts was suggested to indicate their specific role in the coralloid roots (in addition to the fixation of atmospheric nitrogen) [3]. All this shows the importance of the study of the ultrastructure and degradative changes in the heterocysts of symbiotic cyanobacteria.

In the accompanying paper [8], we describe the ultrastructure of the atypical vegetative cells of cyanobionts in the apogeotropic roots of the greenhouse-grown cycads *Cycas circinalis* L., *Ceratozamia mexicana* Brough., and *Encephalartos villosus* Lehm. These unusual forms of vegetative cells with a reduced cell wall (VCRCW) exhibited some specific ultrastructural features indicating that they overproduce either a mucilaginous or proteinous extracellular substance. This indicated that VCRCW may play a specific role in symbiosis.

This paper describes the ultrastructure of heterocysts located close to vegetative cells with a reduced cell wall in the cyanobacterial microcolonies grown symbiotically in the apogeotropic roots of cycad plants.

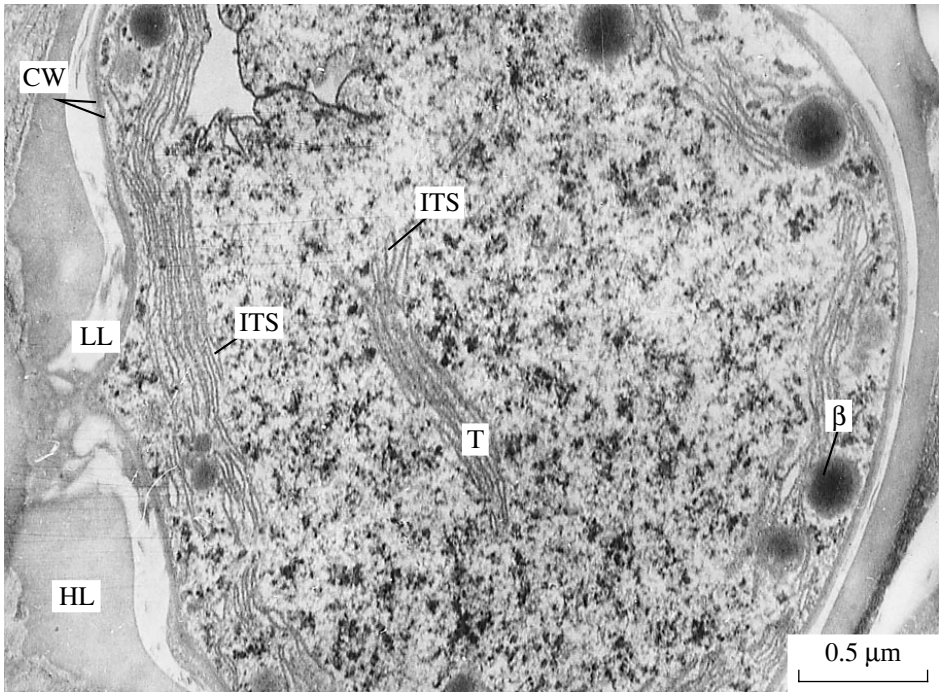

Fig. 3. A cyanobiont heterocyst in the middle region of the coralloid root of *E. villosus*. T, thylakoid; ITS, inner thylakoid space; HL, homogeneous layer of the envelope; LL, lamellar layer of the envelope; CW, cell wall; β, lipid β granule.

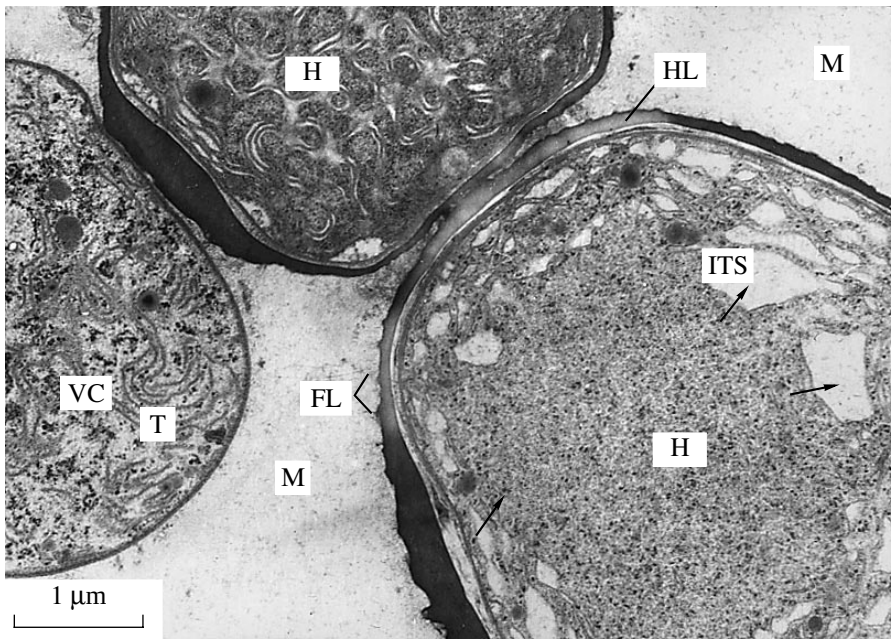

Fig. 4. Heterocysts and a vegetative cell of the cyanobiont in the middle region of the coralloid root of *E. villosus*. The arrows show fibrils in the inner thylakoid space. FL, fibrillar layer of the heterocyst envelope. The other designations are as in the legends to Figs. 1 and 3.

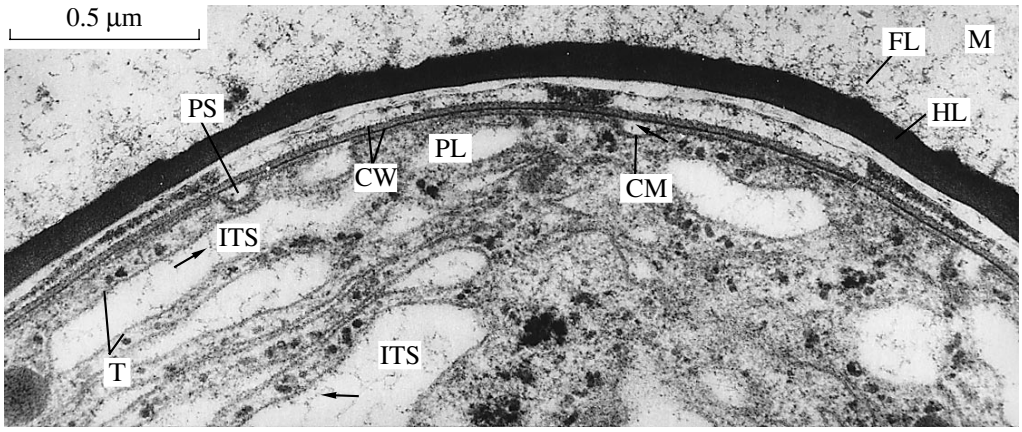

Fig. 5. A fragment of a cyanobiont heterocyst in the middle region of the coralloid root of *E. villosus*. The arrows show fibrils in the inner thylakoid and periplasmic spaces. CM, cytoplasmic membrane; PL, peptidoglycan layer of the cell wall; PS, periplasmic space; OM, outer membrane. The other designations are as in the legends to Figs. 1, 3, and 4.

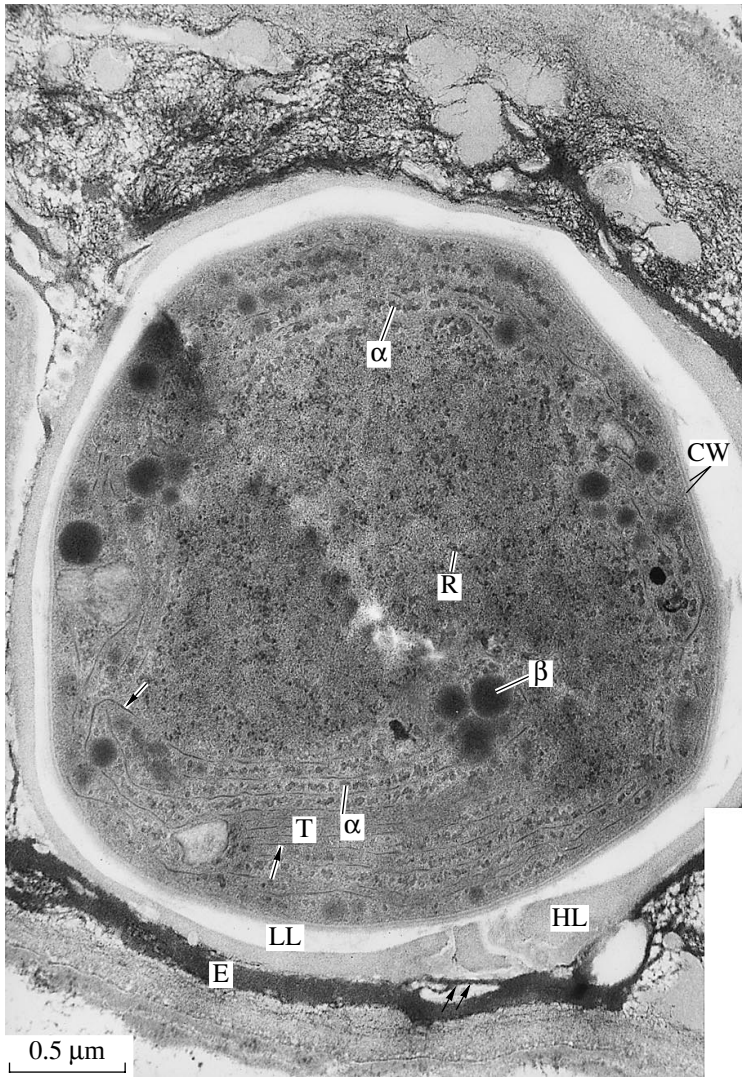

Fig. 6. A cyanobiont heterocyst in the apical region of the coralloid root of *C. mexicana*. One arrow shows a dense zone in the thylakoid interior and two arrows show the pore complex. α, α granules of glycogen; R, ribosome. The other designations are as in the legends to Figs. 2, 3 and 5.

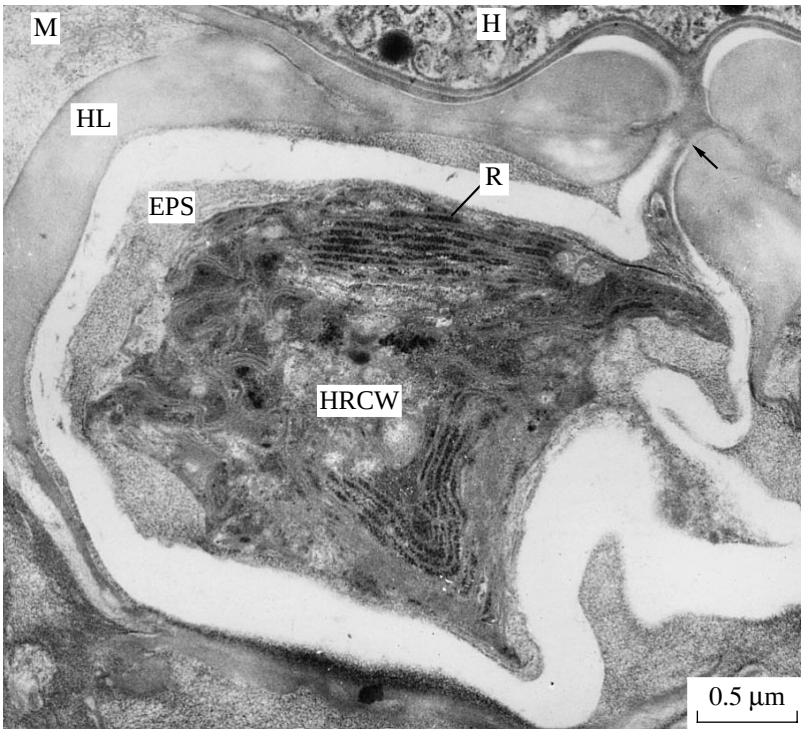

Fig. 7. A contiguous intact heterocyst (H) and a heterocyst with a reduced cell wall in the cyanobiont microcolony grown in the apical region of the coralloid root of *E. villosus.* The arrow shows the region of the merged pore channels of the intact heterocyst and HRCW. EPS, exoprotoplast sheath. The other designations are as in the legends to the previous figures.

MATERIALS AND METHODS

Investigations were carried out with the same samples of apogeotropic roots as were used previously [8]. The samples represented fragments of the coralloids of the *Cycas circinalis* L., *Ceratozamia mexicana* Brough., and *Encephalartos villosus* Lehm. plants grown in greenhouses with subtropical and tropical climates in the Tsitsin Central Botanical Garden of the Russian Academy of Sciences. The *E. villosus* plants were 30- and 50-years-old, and the *C. circinalis* and *C. mexicana* plants were 50-years-old. The root samples were collected in January, April, and June. Root fragments were cut from the apical region (1–3 mm from the apex), middle region (4–10 mm from the apex), and basal region of the apogeotropic roots.

The preparation and electron microscopic analysis of thin sections of roots were carried out as described in the accompanying paper [8].

RESULTS AND DISCUSSION

Electron microscopic studies showed that cyanobiont microcolonies not only had normal heterocysts with the typical ultrastructures of the cytoplasm, the cell wall, and the envelope; but also numerous altered heterocysts with a reduced cell wall (HRCW) (Figs. 1, 2). The altered heterocyst represented a protoplast enclosed in a specialized heterocyst envelope, which showed the ultrastructural manifestations of the overproduction of extracellular substances of a polysaccharide or protein nature, similar to those observed in VCRCW. HRCWs

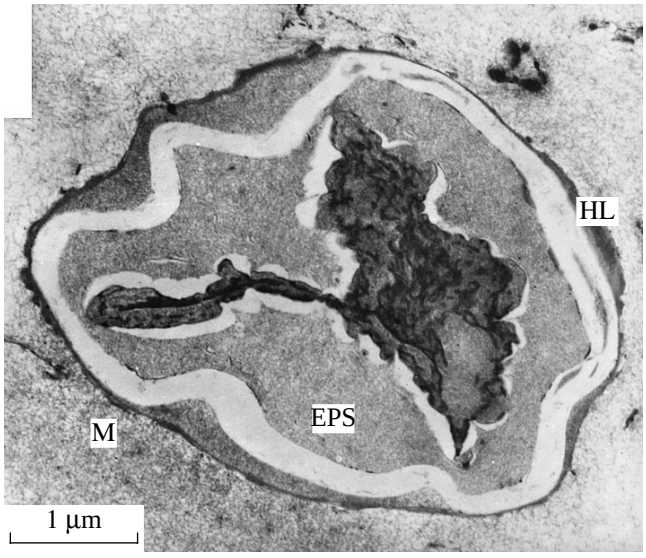

Fig. 8. A cyanobiont heterocyst with a reduced cell wall in the apical region of the coralloid root of *C. circinalis.* Designations are as in the legends to the previous figures.

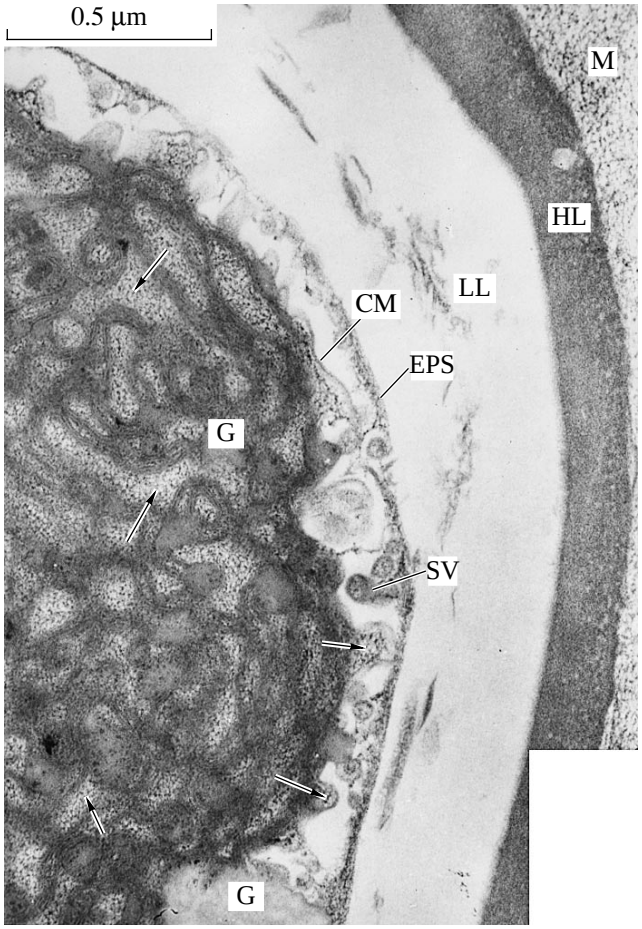

Fig. 9. A fragment of a cyanobiont heterocyst with a reduced cell wall in the basal region of the coralloid root of *E. villosus*. The arrows show the depositions of a fine granular and fibrillar substance in the cytoplasm of HRCW and nascent surface vesicles. G, unidentified granule; SV, surface vesicle. The other designations are as in the legends to the previous figures.

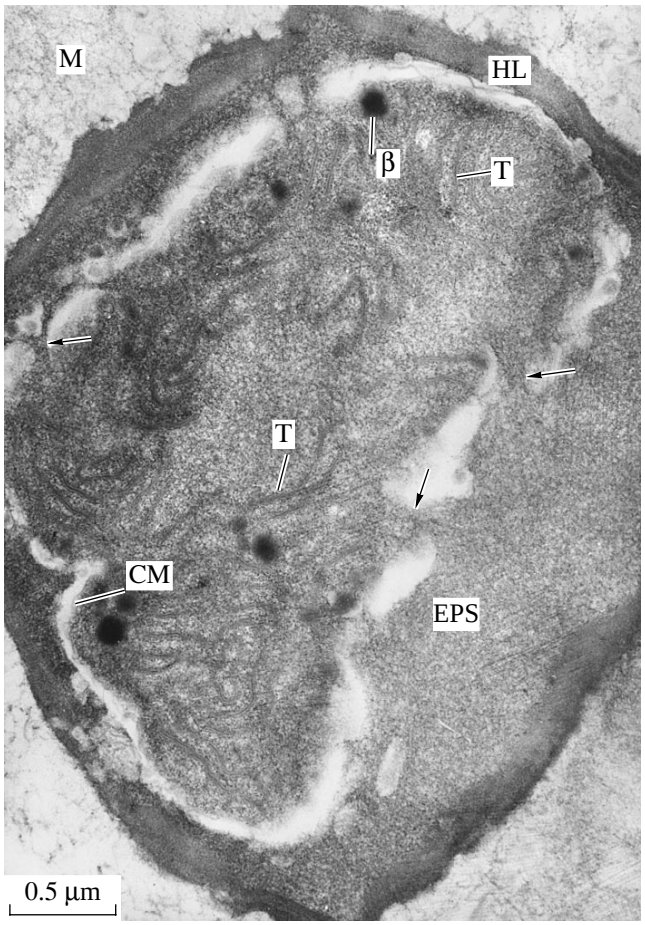

Fig. 10. A cyanobiont heterocyst with a reduced cell wall in the coralloid root apex of *C. mexicana*. The arrows show strands of the fine granular and fibrillar substance in the HRCW cytoplasm merged with the exoprotoplast sheath. Designations are as in the legends to the previous figures.

were detected in the apical, middle, and basal regions of coralloid roots during the spring, summer, and winter periods. The complete lysis of the HRCW cytoplasm was usually not observed, even in the basal region of the apogeotropic roots.

Heterocysts with typical ultrastructure differed from each other in some structural characteristics of their cytoplasm, wall, and especially thylakoids (Figs. 3–6). The ultrastructural characteristics of the thylakoids were similar to those observed for the cyanobionts of *E. transvenosus*, *E. woodii*, and *E. arenarius* plants [4]. For instance, the cyanobionts of *E. villosus* usually had heterocysts with shortened or tortuous thylakoids and a narrow, electron-transparent inner thylakoid space (Fig. 3). At the same time, some heterocysts of the same coralloid roots were found to contain thylakoids with a considerably enlarged interior filled with a fibrillar substance (Figs. 4, 5). Thin fibrils, composed of subunits, were located scarcely and often close to the thylakoid membranes (Fig. 5), suggesting that the latter may be involved in the synthesis of fibrillar polymers. A similar fibrillar substance was observed in the cytoplasmic membrane (CM) invaginations below the peptidoglycan layer (Figs. 4, 5), indicating a functional relationship between the CM and thylakoids. The fibrillar substance formed a distinct layer immediately outside the outer membrane of the cell wall (Fig. 5), producing compact depositions of electron-opaque globules in some regions. The homogeneous layer of the heterocyst envelope was more electron-opaque than it usually is. The outer fibrillar layer and the intercellular matrix were not separated by a boundary.

The swelling of thylakoids in the heterocysts of the cyanobiont of *C. circinalis* was not observed. In contrast, the thylakoid interior was electron-opaque and

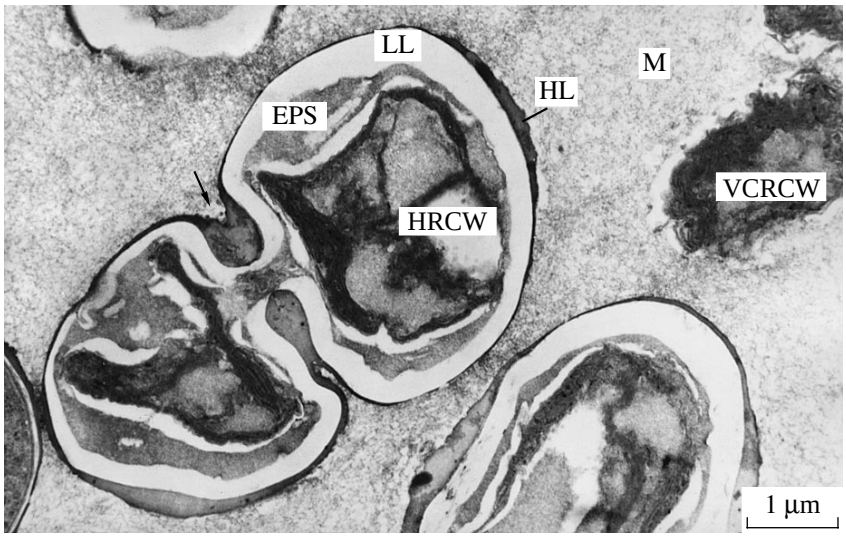

Fig. 11. A fragment of a cyanobiont microcolony in the intercellular space of the cyanobacterial zone of cortical parenchyma near the apical end of the coralloid root of *C. circinalis*. The arrow shows the region of the merged pore channels of contiguous heterocysts with the reduced cell wall. VCRCW, vegetative cell with a reduced cell wall. The other designations are as in the legends to the previous figures.

thick (Fig. 6), which is not typical of cyanobacterial thylakoids. Between thylakoids, there were numerous glycogen granules. The hyaloplasm of these heterocysts was dense and contained substantial numbers of ribosomes and typical cyanobacterial lipid β-granules.

According to our observations, HRCW may dominate in the cyanobiont microcolonies throughout the coralloid root. The morphology of such heterocysts was determined by the configuration of the homogeneous layer of the heterocyst envelope, which was, as a rule, retained in spite of the loss of the cell wall and profound changes in the protoplasts. The latter acquired an irregular shape, either amoeboid or stellate, and often contained long cytoplasmic outgrowths (Figs. 1, 2, 7, 8). These alterations, along with the formation of a fine granular or fibrillar substance resembling the intercellular polysaccharide or the electron-opaque proteinous substance of the cytoplasm and the methods of release of these substances from the protoplast, are analogous to those described for VCRCW [8]. Like the latter cell forms, the altered heterocysts can be divided into two groups, which differ considerably in ultrastructure in accordance with the assumed chemical nature of their extracellular substances.

The HRCWs that produced the substance resembling ultrastructurally the intercellular mucilage were characterized by the presence of a specific sheath in addition to the fibrillar, homogeneous, and lamellar layers of the typical heterocyst envelope. The sheath was located outside the CM and, as a rule, was complementary to the surface of the enclosed protoplast (Fig. 8). The sheath was made of a fine granular and fibrillar substance packaged reticularly similar to how the intracytoplasmic material is packaged (Figs. 8–10, the most demonstrative being Fig. 10). The substance of the sheath was probably released from the cytoplasm with the aid of vesicles pinched off from the surface of the protoplast (Fig. 9), as

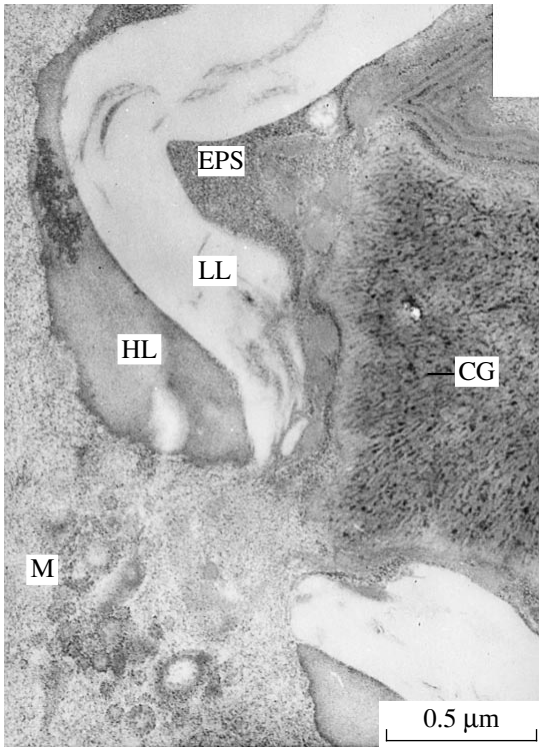

Fig. 12. A fragment of a cyanobiont HRCW near a pore in the middle region of the coralloid root of *E. villosus*. CG, cyanophycin granule. The other designations are as in the legends to the previous figures.

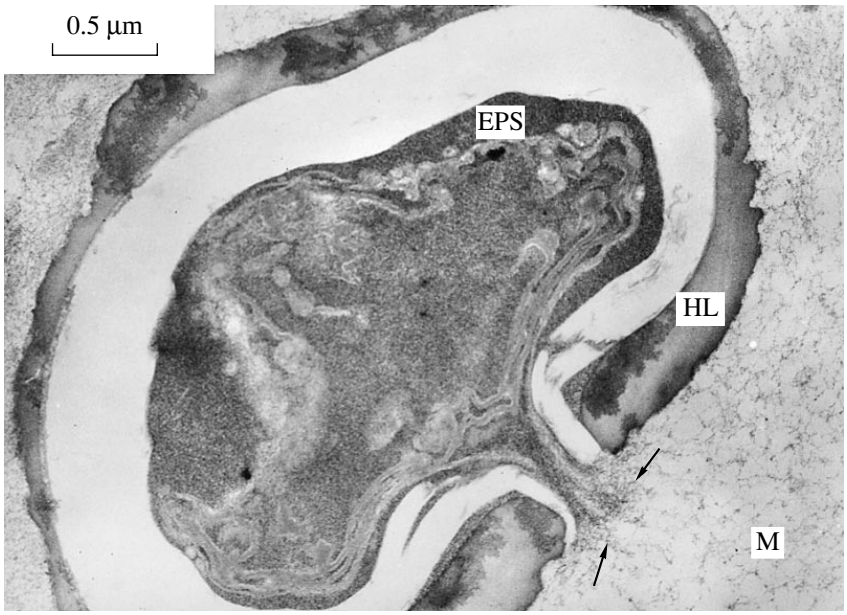

Fig. 13. A cyanobiont heterocyst with a reduced cell wall in the apical region of the coralloid root of *E. villosus*. The arrows show strands of the fine granular and fibrillar substance merged with the mucilaginous intercellular matrix by a pore channel. Designations are as in the legends to the previous figures.

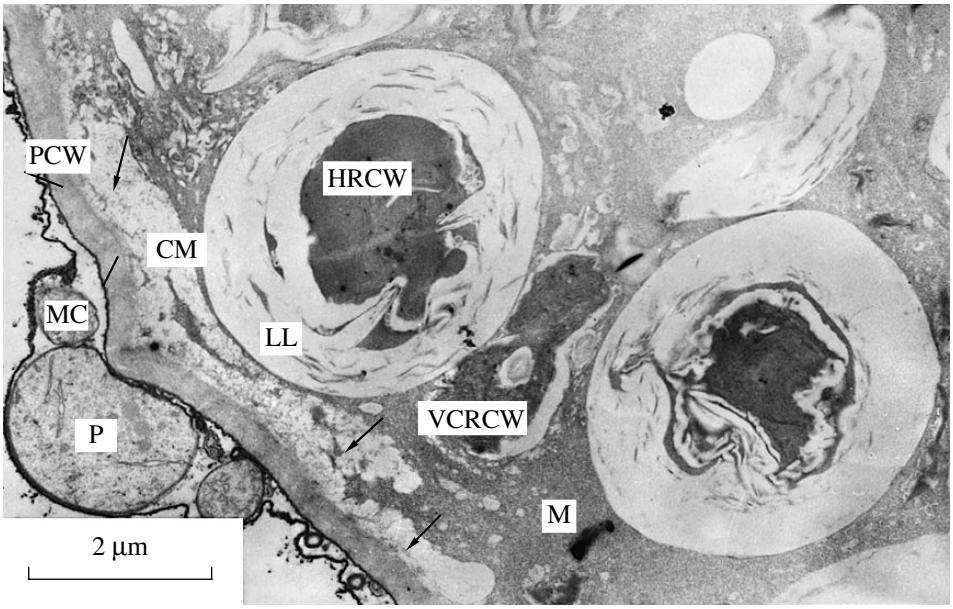

Fig. 14. A fragment of a cyanobiont microcolony in the intercellular space of the cyanobacterial zone of cortical parenchyma in the middle region of the coralloid root of *C. mexicana*. The arrows show the interface between the mucilaginous intercellular matrix and the plant cell wall. TP, tonoplast; P, plastid; MC, mitochondrion. The other designations are as in the legends to the previous figures.

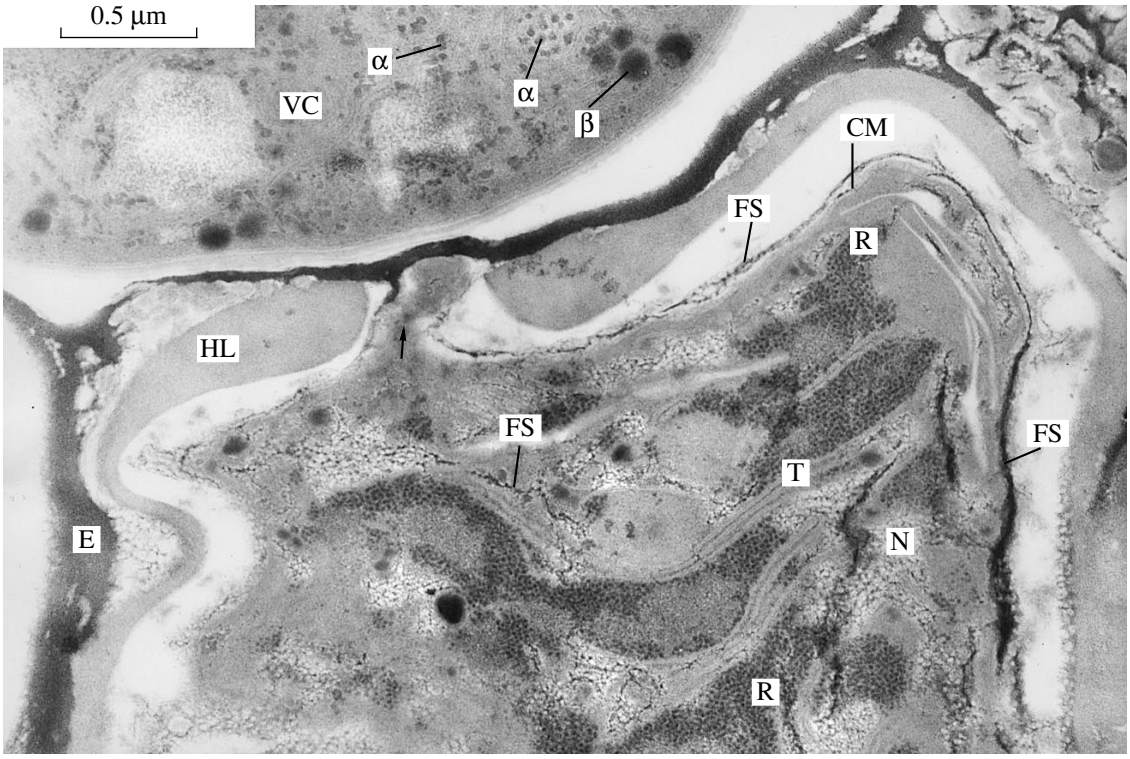

Fig. 15. A fragment of a cyanobiont HRCW contiguous with a vegetative cell in the apical region of the coralloid root of *C. mexicana*. The arrows show the pore complex. FS, electron-opaque fibrillar substance; N, nucleoid. The other designations are as in the legends to the previous figures.

well as by local ruptures in the CM (Fig. 10). In the latter case, as can be seen from Fig. 10, the cytoplasm is linked with the protoplast-enclosing sheath. The substance of the sheath may penetrate through the pore local ruptures to adjacent altered heterocysts (Fig. 11), intact heterocysts (Fig. 7), or mucilaginous intercellular matrix (Figs. 12, 13). This suggests that HRCWs, like VCRCWs, are involved in the formation of intercellular mucilage. As can be seen from Figs. 1 and 14, there is a well-defined boundary between the intercellular mucilage and the plant cell wall. The cyanobionts located in the middle region of the coralloid roots of *C. mexicana* (samples for this analysis were taken in January) contained altered heterocysts, which were spherical in shape and had a hypertrophic inner lamellar layer but lacked the homogeneous layer of the envelope typical of normal heterocysts (Fig. 14).

The thylakoid membranes of altered heterocysts with additional exoprotoplast sheaths were usually closely pressed together. Between thylakoids, there was a material ultrastructurally resembling that of the intercellular matrix and the sheaths observed on the micrographs of ultrathin sections contrasted with lead citrate and uranyl acetate. Ribosomes and nucleoid zones typical of cyanobacteria were not usually observed, although the inner thylakoid space was sometimes filled with regularly arranged ribosomes (Fig. 7). The cytoplasm of the altered heterocysts contained, although rarely, typical cyanobacterial lipid β-granules (Fig. 10) or electron-transparent granules of an unknown nature (similar to those observed in the VCRCW) (Fig. 9), or large cyanophycin granules located in the vicinity of pores (Fig. 12).

The ultrastructure of the HRCW of the cyanobiont of *C. circinalis* (these HRCWs produce a protein-like substance) was characterized (in addition to the presence of several nucleoid zones) by numerous regularly arranged ribosomes and the deposition of an electron-opaque substance in the cytoplasm, outside the CM, and on the periphery of a neck-shaped protoplast outgrowth located near the pore and the homogeneous layer of the heterocyst envelope (Fig. 15). The electron-opaque substance merged with the additional outer layer of altered heterocysts, as in the case of intact heterocysts (Fig. 6) and the other cell forms of symbiotic microcolonies (Fig. 2).

Thus, the ultrastructural study of the cyanobionts of different cycad genera and species showed that the differentiation of heterocysts is likely to be associated with changes in the regulatory mechanisms which trigger or enhance the intracytoplasmic synthesis of extracellular substances not specific to the heterocysts.

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