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EXPERIMENTAL ARTICLES

Infection of Plants and Plant Tissue Cultures with Cyanobacteria–Bacteria Complexes

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Abstract—The infection of tobacco, nightshade, rice plants, and their tissue cultures with the cyanobacteria–bacteria associative microsymbiont complexes (AMC) isolated from natural syncyanoses (the ferns *Azolla pinnata* and *Azolla* sp. and the cycad *Encephalartos ferox*) was studied. The inoculation of the intact plants or their cuttings with AMC led to the colonization of the plant roots, stems, and leaves by cyanobacteria and their bacterial symbionts (referred to as satellite bacteria, SB). The sites of the long-term contact of plant organs with cyanobacteria were characterized by the formation of copious slime. On the roots of infected plants, one could observe the callus growth of cortical parenchyma cells and the formation of pseudonodules, in which SB cells gradually accumulated. In mixed cultures of plant callus tissues and the AMC isolated from the ferns *A. pinnata* and *Azolla* sp., the callus tissue specifically influenced the growth of the AMC components, causing (depending on the plant species and strain) either their balanced growth, or their cyclic growth, or the predominant growth of one of the AMC components (either cyanobacteria or satellite bacteria). This phenomenon is proposed to be used for the dissociation of stable multicomponent natural symbiotic complexes and the selection of their particular components.

Key words: model associations, cyanobacteria, satellite bacteria, plant infection, callus cultures.

In our earlier studies, the model associations of different plant objects (cell cultures, tissues, and cuttings) with free-living cyanobacteria were created [1]. Stable plant–cyanobacteria associations resulted, as a rule, from plant regeneration in a mixed callus culture [2, 3].

The formation of syncyanoses in nature usually includes the stage of the primary infection of plant tissues and organs by cyanobacteria [3]. Experiments with the isolation of cyanobionts from natural plant syncyanoses and the reconstitution of the latter with the use of symbiotic and free-living cyanobacteria showed that many of them are not specific for particular plants: free-living cyanobacteria of the genus *Nostoc* were found to be able to form stable associations with the mosses *Anthoceros punctatus* [5] and *Phaeoceros laevis* [6], the cycad *Zamia furfuracea* [7], and the angiosperm *Gunnera manicata* [8].

The cyanobacteria–bacteria associative microsymbiont complexes (AMCs) and symbiotic cyanobacteria (cyanobionts) isolated from host plants can be cultured under laboratory conditions [9] and may be more active than free-living cyanobacteria with respect to the infection of nonsymbiotrophic plants and their organs and tissues.

The aim of the present work was to study the ability of the cyanobacteria–bacteria symbiotic associations isolated from the ferns *Azolla pinnata* and *Azolla* sp. and the cycad *Encephalartos ferox* to infect plants and plant callus cultures.

MATERIALS AND METHODS

Axenic plants of the rice *Oryza sativa* L. cv. Kuban'-3, the tobacco *Nicotinia tabacum* L. cv. Samsun, and the black nightshade *Solanum nigrum* L. were grown from seeds sterilized with 30% hydrogen peroxide for 30 min. The plants were grown on solid medium containing mineral components (as recommended by Murashige and Skoog [10]) and 0.7% agar. Rice seeds were obtained from the Russian Research Institute of Rice in Krasnodar. Nightshade seeds were collected in the Botanical Garden of the Moscow State University. Axenic plants of the albino plastom tobacco mutant *N. tabacum* P1 cv. Samsun were obtained from the Department of Cultured Cells of the Timiryazev Institute of Plant Physiology of the Russian Academy of Sciences.

Cuttings of the wild-type and mutant tobacco plants and nightshade plants were prepared and grown as described in the handbook [11]. The growth medium for the mutant *N. tabacum* P1 cuttings contained 4% sucrose.

Primary callus from the rice seeds and germs was prepared as described by Kucherenko [12]; the tobacco and nightshade callus tissues were prepared from the leaf explantants of 1-month-old axenic plants [11]. The callus tissues were cultured in media modified according to the specific requirements of particular plants [11].

The cyanobacteria–bacteria symbiotic associations isolated from the ferns *A. pinnata* and *Azolla* sp., and the

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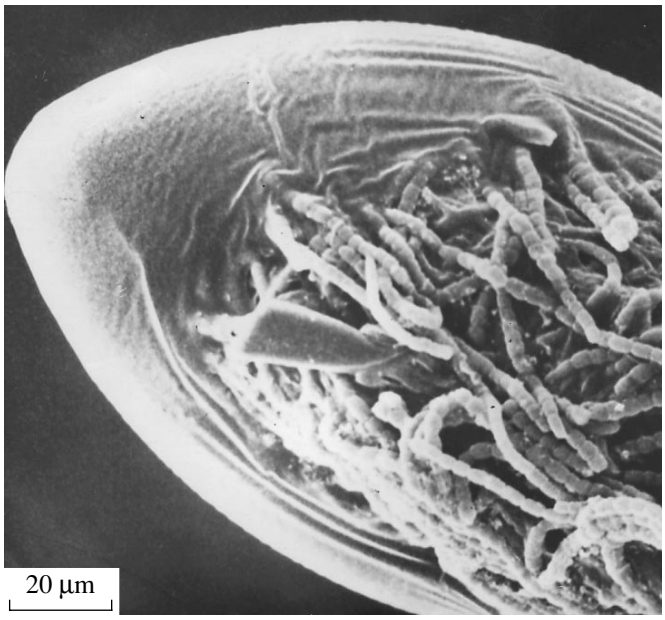

Fig. 1. Cyanobacteria from the *E. ferox* AMC submerged in slime on the surface of the rice root cap.

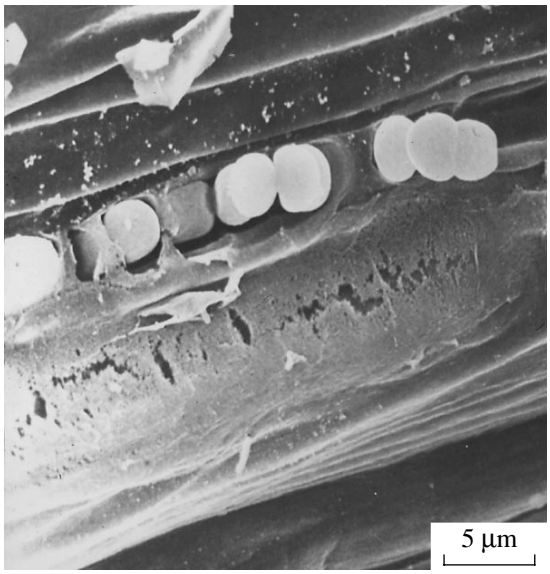

Fig. 2. Nightshade root fragment: chains of cyanobacteria from the *E. ferox* AMC submerged in slime in the root depression.

cycad *E. ferox* were cultivated as described earlier [9]. The AMC included symbiotic cyanobacteria (or cyanobionts) and satellite bacteria (SB).

Rice plants and the nightshade and tobacco (both wild-type and mutant) plants were inoculated with AMC by placing 1 ml of 1-month-old AMC suspension on the surface of agar medium near the plants or cuttings. The concentration of cyanobiont in the AMC suspension was 6.25×10^5 cells/ml.

The rice, tobacco, and nightshade calluses were inoculated with AMC by placing 1 ml of the same AMC suspension on the surface of the calluses during their transplantation.

Specimens for light and electron microscopy were prepared as described elsewhere [13].

RESULTS AND DISCUSSION

Infection of whole plants. After the inoculation of rice, tobacco, and nightshade plants, as well as of tobacco and nightshade cuttings, with AMC, symbiotic cyanobacteria were detected on the surface of plant roots, stems, and leaves. On the roots, cyanobacteria were localized in the zones of cell division (beneath the root cap) (Fig. 1), cell elongation (Fig. 2), and root crown in the form of ascending threads. On leaves, they were localized in depressions on the leaf surface (Fig. 3) and at the leaf edges (Fig. 4), forming a thin amorphous net (Fig. 5) or individual microcolonies near a. Moreover, cyanobiont threads penetrated into the leaf interior through stomatal openings (Fig. 6).

It should be noted that the earlier studies of the model association of the tobacco cv. Samsun mosaic mutant callus tissue and the free-living cyanobacterium *Anabaena variabilis* ATCC 29413 showed that the cyanobacterial cells were able to reach the leaf exterior of the tobacco plants through the intercellular space and xylem vessels (which are common to the plant and callus tissues) and then through the air-conducting stomatal openings [13]. Taking into account these data, we may suggest that, when whole plants are inoculated with AMC, symbiotic cyanobacteria and SB can penetrate into plant tissues through stomatal openings and colonize intercellular space in all plant organs.

In the roots of AMC-inoculated plants, symbiotic cyanobacteria and SB caused tissue dedifferentiation and the formation of pseudonodules (or *para*-nodules) (Fig. 7). The microscopic examination of the root region with the pseudonodules revealed zones with the callus growth of the actively proliferating cells of cortical parenchyma.

Degrading cyanobacterial cells were detected in the intercellular space of the peripheral zone of pseudonodules, whereas SB cells were detected in the intercellular space of the central part of the pseudonodules; in this case, many SB cells were separated from the plant cell walls with an electron-dense layer (Fig. 8). Bacterial cells in the intercellular space of pseudonodules were surrounded by a great number of electron-dense granules 30 to 90 nm in size (Fig. 9), produced most likely by the bacterial cells. These observations indicated that the SB cells of the plant–bacterial associations were functionally active.

The inoculation of rice plants and tobacco and nightshade cuttings with AMCs stimulated rhizogenesis: the total length of the AMC-inoculated rice and

tobacco plant roots was, on the average, 20% longer than that of the control plants.

The depressions on the surface or roots and stems colonized by cyanobacteria contained slime (Figs. 2 and 3). In the course of time, the slime became more viscid and formed a mantle with submerged cyanobacterial threads (Figs. 2 and 3). The occurrence of cyanobiont in slime on the surface of plant organs seems to provide for the close contact between cyanobacteria and the plant tissue and thus facilitate the exchange of metabolites between them. The production of copious slime is typical of all the known natural syncyanoses [4]. In the artificial associations of free-living cyanobacteria with plants, slime, composed of acidic polysaccharides, was also produced on the surface of callus and tissue cultures, plant regenerates, and plant roots [1, 13]. The slime was produced by both plants and bacteria.

There was no visible growth of SB on the surface of the inoculated plants or in the cultivation medium.

The ability of AMC isolated from the ferns *A. pinnata* and *Azolla* sp. to infect plant callus cultures. The inoculation of the rice, nightshade, and tobacco mutant P1 calluses with the AMC led to the formation of mixed culturable calluses.

The callus tissues of the tobacco mutant P1 and nightshade were light yellow, soft, loose, and were found to be formed by parenchymal cells. When the callus tissues were inoculated with AMC, symbiotic cyanobacteria colonized them uniformly, so that 1 month after inoculation, the callus tissues turned emerald-colored. Cyanobacterial cells propagated inward the calluses through the intercellular space. The resultant mixed calluses were maintained by transferring their 1×1 cm pieces to fresh nutrient medium at 18- to 20-day intervals.

The examination of the mixed calluses in a scanning electron microscope showed the presence of a small number of SB cells closely contacting cyanobacterial cells (Fig. 10). There was no visible growth of SB or cyanobacteria in the cultivation medium.

Unlike the tobacco and nightshade calluses, the rice callus was yellow, uneven, hard, dense, and was formed by the closely packed parenchymal cells. Within the first 20 days after the callus inoculation with AMC, cyanobacteria grew in the callus fractures in the form of brightly colored, granular, dull colonies. Cyanobacterial cells were unable to penetrate into the callus tissue, so that cyanobacterial colonies could easily be separated from the callus. After 20 days of incubation, the diameter of the colonies changed little, but dull colonies gradually turned shiny and colorless due to the formation of copious transparent slime on their surface. Further incubation led to the formation of the dull colonies of SB cells on the surface of the slimy cyanobacterial colonies.

The mixed rice callus was maintained by transferring its pieces with cyanobacterial or SB colonies to fresh nutrient medium at 25- to 30-day intervals. Soon after the transfer, the region of the callus with the colonies turned intensively green-colored due to the rapid growth of cyanobacteria. The growth of AMC in association with the rice callus was similar to its growth on mineral agar media described earlier [9]. The growth of

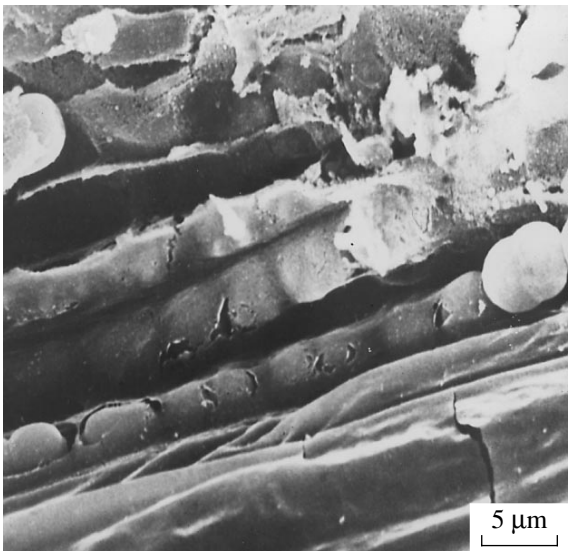

Fig. 3. A fragment of the mutant tobacco P1 stem: threads of cyanobacteria from the *A. pinnata* AMC submerged in slime in the surface wrinkles.

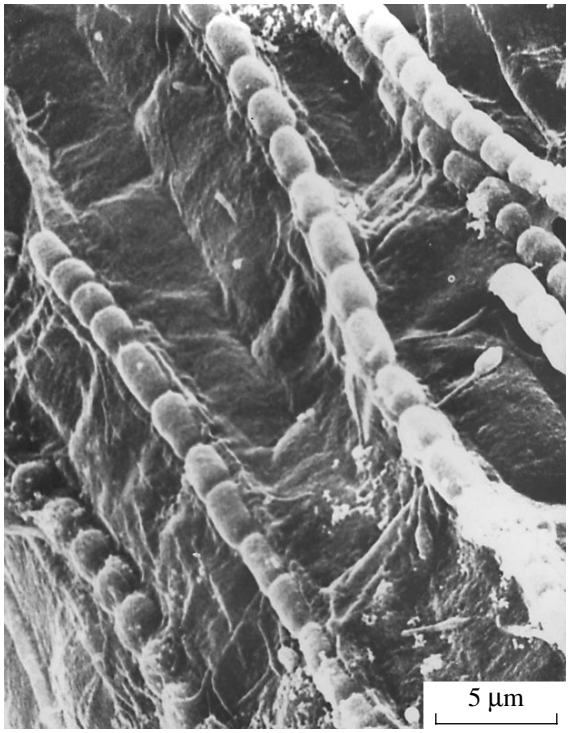

Fig. 4. Nightshade stem fragment: cyanobacterial chains located at the leaf edges.

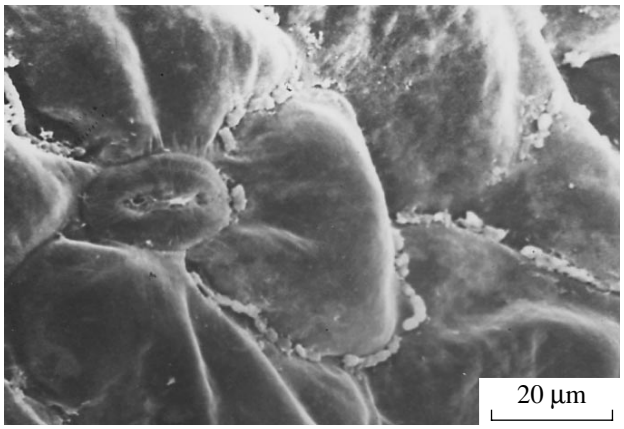

Fig. 5. Threads of cyanobacteria from the *A. pinnata* AMC on the surface of the albino mutant tobacco P1 leaf.

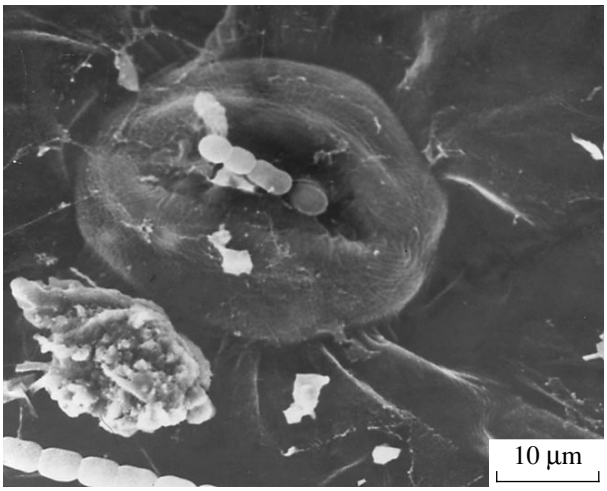

Fig. 6. Nightshade leaf fragment: cyanobacteria in a stomatal opening.

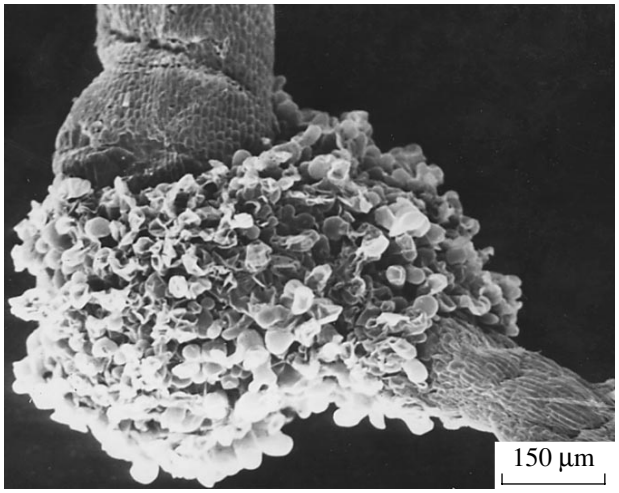

Fig. 7. Nightshade root fragment: the appearance of a pseudonodule.

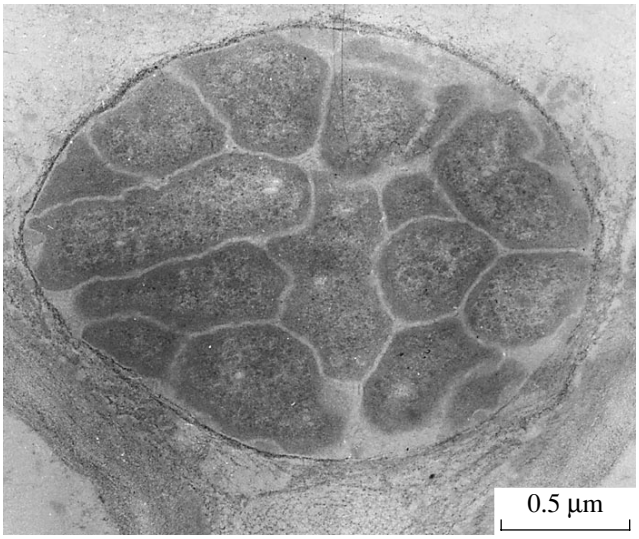

Fig. 8. SB cells in the intercellular space of a pseudonodule.

SB was observed not only on the callus surface, but also in the cultivation medium in the vicinity of the callus.

The structure of the wild-type tobacco callus tissue was similar to that of the mutant P1 callus tissue. However, the inoculation of the wild-type tobacco callus with AMC did not lead to the formation of mixed culturable callus: cyanobacteria could grow on the callus surface in the form of colonies in the first 14 days of incubation, but then the colonies turned colorless and disappeared. After 18–20 days of incubation, smooth and shiny SB colonies appeared on the callus surface. The intense growth of the SB colonies on the callus surface and SB cells in the cultivation medium led to the rapid death of the callus.

The differences in the colonization patterns of the tobacco, rice, and nightshade calluses with AMC may be due to their different structures. In particular, the hard and dense rice callus tissue almost lacks intercellular space, where cyanobacteria would undergo symbiotic transformations [13]. On the other hand, the interaction patterns of AMC with the callus tissues of the wild-type and mutant strains of tobacco cv. Samsun are different, although these calluses have similar structure. This observation is not surprising, since even cell or tissue cultures derived from the same plant may considerably differ from each other [11]. Therefore, the infection patterns of plant tissue cultures depends on their morphological and metabolic features and is not only species-specific but even strain-specific.

SB cells could grow on the surface of only senescent mixed calluses and pseudonodules (Fig. 11); in this case, the growth of smooth and shiny SB colonies was

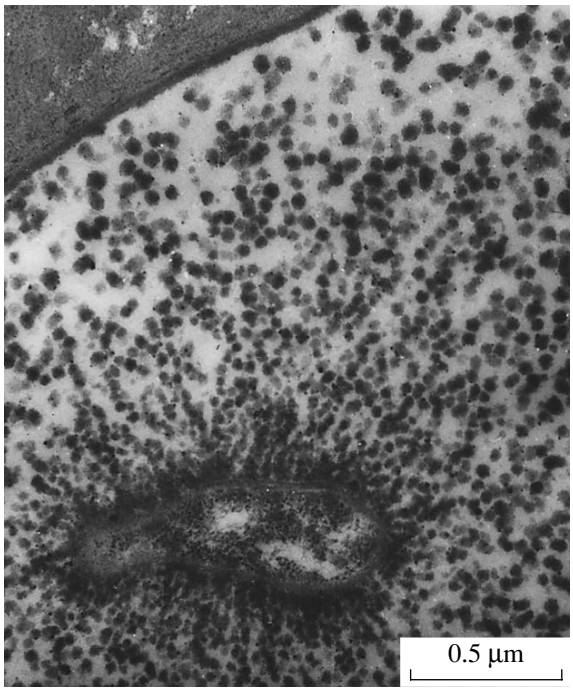

Fig. 9. Granules of an electron-dense substance surrounding SB in the intercellular space of a pseudonodule on the mutant tobacco P1 root.

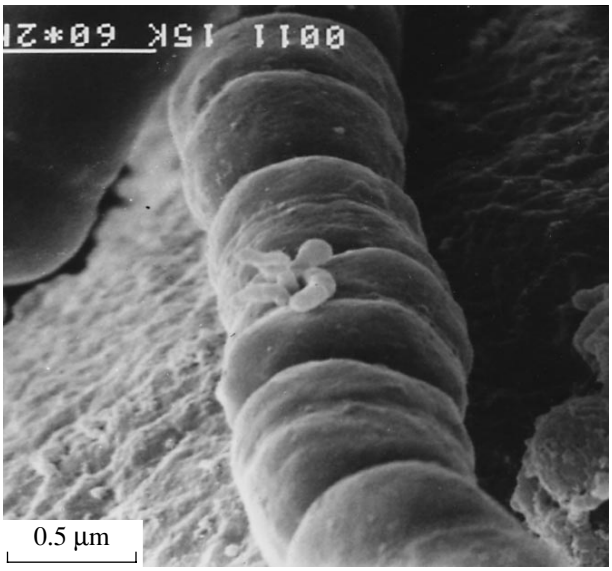

Fig. 10. SB cells of the *Azolla* sp. AMC on the surface of a cyanobacterial colony in a mixed culture with the mutant tobacco P1 callus.

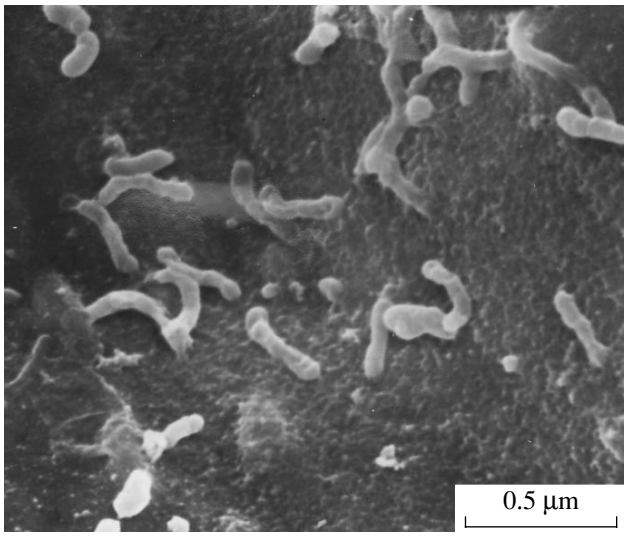

Fig. 11. SB cells on the surface of the senescent nightshade callus.

associated with the degradation of the mixed calluses and the decolorization of cyanobacterial colonies.

The colonization pattern of rice plants and the rooted cuttings of nightshade and tobacco (both wild-type and mutant) by AMC considerably differed from the colonization pattern of the callus cultures derived from these plants. Earlier studies of the model associations of plant tissues with free-living cyanobacteria showed that their growth has two phases: the growth of plant tissues in such associations is primary, while cyanobacteria begin to grow only in the stationary growth phase of calluses [14, 15]. In the present study, plant calluses were found to exert specific effects on the growth of AMC as a whole and of its particular components. During the mixed cultivation of the *Azolla* sp. or *A. pinnata* AMC with calluses, the AMC components grew differently, although their growth was balanced in the absence of the calluses [9]. Depending on the plant species or strain (in the case of tobacco), the mixed cultivation of AMC with the calluses was accompanied by the predominant growth of one of the AMC components or their cyclic growth (Fig. 12). The different growth patterns (balanced, cyclic, and dominant) of cyanobacteria and SB in the mixed cultures of AMC with the plant callus tissues can presumably be used for the separation of stable, multicomponent natural symbiotic complexes into components and their subsequent selection. It should be noted that SB cells selectively accumulate in the pseudonodular tissues of laboratory plants throughout their life.

The culturable associations of the tobacco and nightshade calluses with AMC may be considered promising models for the long-term investigation of the symbiotic properties of microsymbionts.

The ability of AMC to efficiently infect various plant organs and to induce the formation of root pseudonodules is probably due to the activity of symbiotic SB. One of the possible functions of SB in the *Azolla* syncyanosis is the synthesis of indole-3-acetic acid (IAA) [17]. The formation of pseudonodules on the root of nonlegume plants is presently considered to be one of the most promising methods to render them capable of nitrogen fixing [18]. To induce pseudonodu-

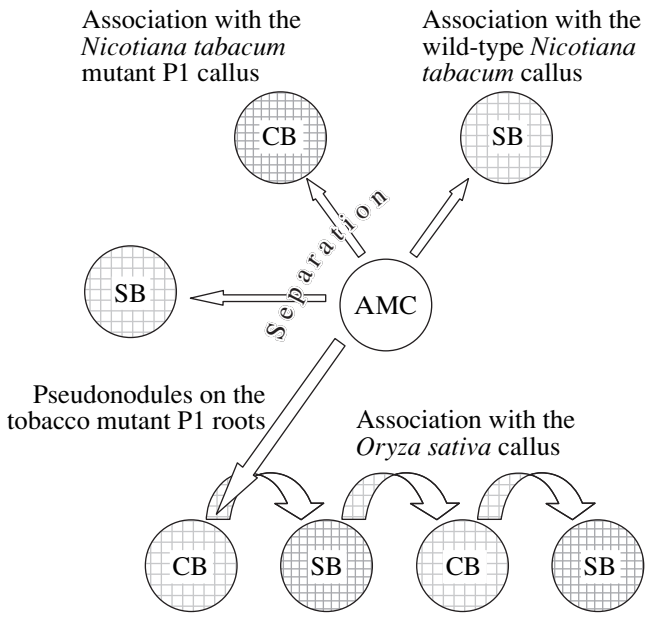

Fig. 12. Schematic representation of the interaction patterns of AMC and its components with the cultivated plant tissues. CB, cyanobacteria; SB, satellite bacteria; and AMC, cyanobacteria–bacteria symbiotic association.

lation, researchers use natural auxins (such as IAA) or their synthetic analogues (2,4-D, chlorambene, and chlorosulfonic acid) [19]. In the associations described here, AMC induced the formation of pseudonodules in which metabolically active SB cells gradually accumulated. The callus-like tissue of the pseudonodules may promote the penetration into the plant tissue and the propagation therein not only of cyanobacteria, but also of other active diazotrophic bacteria. The revealed possibility of the AMC separation into particular components (cyanobacteria and SB) and their subsequent selection shows the prospects of the creation of artificial associations of free-living cyanobacteria with the SB isolated from natural symbiotic associations.

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