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EXPERIMENTAL ARTICLES

Cyanobacterium *Nostoc paludosum* Kütz as a Basis for Creation of Agriculturally Useful Microbial Associations by the Example of Bacteria of the Genus *Rhizobium*

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Abstract—Different species of *Rhizobium* were successfully introduced into the extracellular slime of *Nostoc paludosum* (Kütz) Elenk, strain 18; cyanobacteria did not eliminate them and exhibited no specificity to the introduced species. Both partners were shown to exist in a self-sufficient manner in an artificial consortium, the stability of which is determined by the technology of growing the cultures in collections. Cyanobacteria act as carriers of introduced satellites, providing contact with the inoculated material through the slime, and increase the nitrogen-fixing ability of legume plants due to the increase of the number and activity of nodules. The fact of penetration of cyanobacterial hormogonia into the nodules has been noted. The treatment of seeds by the consortium resulted in an increase of the harvest as compared with the standard methods of nitragin treatment of legumes.

Key words: cyanobacteria, root nodule bacteria, cyano-rhizobial consortia, effect on plants.

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The presence of slime satellites in cyanobacteria (CB) in all of the ecotopes studied is a fact acknowledged by all soil microbiologists, hydrobiologists, and ecologists [1, 2].

It has been established that the slime satellites of CB vary both in quantity and in species diversity; their composition may vary depending on the ecotope features. It seems that to some extent CBs recruit the bacteria which are necessary for their existence in a given ecological niche [3]. This is probably one of the reasons for the high adaptive capacities of CB. At the same time, attempts to remove satellite bacteria from the extracellular slime inevitably weaken the culture to the point of loss of viability [3, 4]. At the same time, algologically pure CB cultures, containing populations of various saprotrophs, are maintained in collections for decades.

All the above opens prospects for construction of novel microbial associations by removal of CB natural satellites and introduction of programmable microflora. The application of CB is attractive, because these organisms are autotrophic nitrogen-fixers and the cost of their cultivation can therefore be decreased. Their high capacities of adaptation to the fluctuations of hydrothermal regime and soil conditions are also attractive.

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The goal of the present work was creation of cyano-rhizobial consortia (CRC) by means of introduction of *Rhizobium* bacteria into the cellular slime of CB to enhance the efficiency of the standard method of nitragin treatment of legumes.

For the purpose of using CB as nitrogen fixers and growth stimulators, the task of protection of the introduced selective rhizobial strains from indigenous microflora, from unfavorable conditions of soil environment, and from adhesion of inoculum to the treated material needed to be performed.

MATERIALS AND METHODS

The culture of cyanobacterium *Nostoc paludosum* (Kütz) Elenk, strain 18, maintained in the collection of the Vyatka State Agricultural Academy by the standard methods for alcoholically pure cultures, was used in the work [5]. This cyanobacterium was selected in accordance with the screening of collection cultures due to its high rate of nitrogen fixation, formation of biomass, and technological effectiveness of cultivation [6, 7]. Every 2–3 months, the strain was transferred on fresh agarized Gromov medium no. 6 without nitrogen [8]; it was maintained at a temperature not above 10°C on slants in test tubes. To obtain bulk cultures, *Nostoc* was inoculated in 100–250-ml Erlenmeyer flasks and cultured in a luminostat at 2000–3000 lx. The 1–1.5-month CB cultures

were used depending on the objectives of the experiment. The culture purity was determined by direct microscopy and by inoculation on selective media.

The bacterially pure culture of *N. paludosum* was available. Axenization of the culture was performed according to the methods described previously [6] and included stepwise the maximum possible removal of cyanobacterial slime by filtration of the culture through asbestos filters fixed in Allen funnels and UV irradiation of CB by a PRK-4 lamp at 30 cm for 2 min. This procedure did not result in an axenic culture but provisionally reduced the titer of satellite bacteria and eliminated the fungi. The fundamental difference of our technology from the generally accepted one, consisting in the frontal attack of antibiotics on satellite bacteria [9, 10], is the targeted action on particular satellite microorganism. Bacterial satellites were transferred from selective media and grown as an even lawn on the same medium in a petri dish. Then, antibiotic test rings with a set of 8 antibiotics were placed onto this lawn (Antibiotika-Testringe DBGMG 7343614; "HEINRICH MACK NCHF. CHEM."). The most efficient antibiotic was chosen by the size of the zone of lysis of tested bacteria. The antibiotic was then introduced into the purified CB culture for elimination of particular species of bacterial contaminant. Similarly, the activity of antibiotics against other satellite microbes was tested and the latter were removed from cyanobacterial cultures as antibiotic-sensitive targets. The antibiotic concentration was adjusted so that CB cells remained viable. CB axenicity was controlled by inoculation on selective media (nutrient agar, Ashby, starch–ammonium agar, etc.) and direct microscopy.

The compatibility of axenic CB culture with root nodule bacteria (RNB) was studied on the following strains obtained from the collection of the All-Russian Research Institute of Agricultural Microbiology (Pushkin, St. Petersburg): *Rhizobium leguminosarum* (Frank 1879) Frank 1889 strain 1022, *Rh. galegae* Lindström 1989 strain 0702, *Rh. trifolii* Dangeard 1926 strain 348a, and *Mesorhizobium loti* Jarvis et al. 1982 strain 1801. These strains had passed preliminary testing at the All-Russian Research Institute of Agricultural Microbiology for the efficiency of inoculation of the corresponding legumes [11, 12]. Rhizobial cultures were maintained on 2% agarized bean broth with 20 g of sucrose per liter of distilled water [13].

The interaction of the partners within cyano-rhizobial consortia (CRC) was studied by inoculation of RNB suspension with the initial titer of $1\ldots3 \times 10^9$ cells/ml (by turbidity standard) to the 1-month axenic culture of *N. paludosum*. CB growth was monitored by an increase of dry matter.

The quantity of RNB in cocultures was controlled by inoculation of appropriate dilutions of CRC culture liquid on agarized (2%) bean broth.

The procedure of photography and the indicators of reality of artificial consortia with rhizobia have been described previously [14, 15]; the photographs necessary for further discussion of the material are presented in the text.

Root nodules were analyzed by their quantity, size, and color [16]. Garden peas (*Pisum sativum* L.) of the Nadezhda and Alpha varieties were used in the work.

The experiments in water cultures were performed according to the classical procedure [17] on Knop medium. Inoculation was made in two weeks after the planting of pea germs.

During sampling, the nodules were cleaned from cyanobacterial surface overgrowth using a squirrel brush, centrifuged, and repeatedly resuspended in a fresh Knop solution. Simultaneously, the absence of CB filaments attached to the nodule surface was controlled microscopically. The washed nodules were fixed in 4% formalin. Thin cross sections of the nodules were microscoped at ×1350; for photographing, the picture was additionally magnified five times using the "optical zoom" function; for printing, fourfold digital magnification was used.

The rhizogenic effect was determined by the length and volume of roots (measured by water displacement). The growth of above-ground part of plants was checked by measurements and determination of dry matter.

The coefficient of symbiotic nitrogen fixation was calculated in a vegetation experiment with meadow clover (*Trifolium pratense* L.) by comparison with a non-bean culture [18]; nitrogenase activity in the nodules together with root fragments was determined by the modified method of the All-Russian Research Institute of Fodder [19]. In order to make certain that inoculation proceeded due to experimental rhizobial strains, the latter were labeled by str^r and rif^r. The labeled strains were obtained from the All-Russian Research Institute of Agricultural Microbiology. The quantity of CRC cells adhered to the seeds was determined by direct counting after washout.

RESULTS AND DISCUSSION

The obtained axenic CB culture was used for studying the possibility of its existence along with the microbial colonizers applied in the experiment. Different species and strains of *Rhizobium* bacteria were added to the pure CB culture in an attempt to increase the resistance of rhizobial cells inoculated to legume seeds. As a result, an artificial stable CRC was created.

We have previously shown that the colonizer is not removed from the CRC of *N. paludosum* 18 and *Rh. leguminosarum* 1022 after repeated washing with centrifugation and resuspension of CRC into a fresh medium, which confirms the affinity between partners [7].

Figure 1a shows the arrangement of rhizobial cells in the slime of CB along their filaments. Rhizobia penetrate the slime, probably due to chemotaxis; the products of carbon dioxide and dinitrogen fixation secreted by cyanobacterial cells possibly acted as attractants (penetration of bacteria into the slime was found to

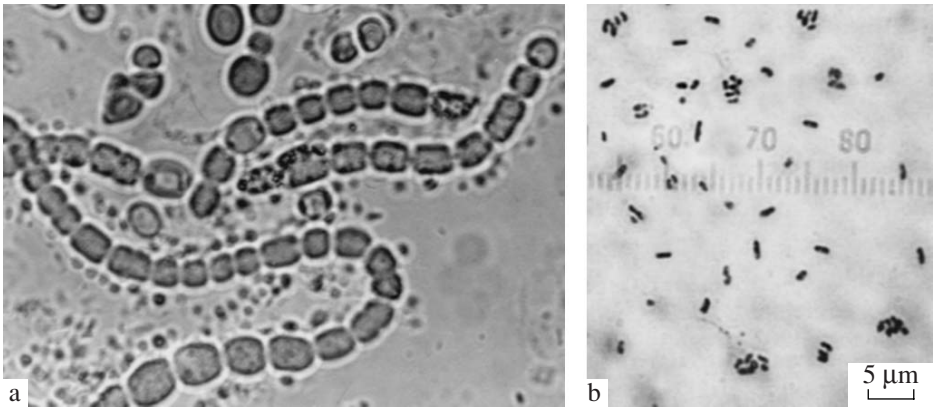

Fig. 1. Mucigel surrounding the vegetative cells of *N. paludosum* and containing *Rh. leguminosarum* bacteria (a); phase contrast, ×2150; *Rhizobium* on bean medium (b).

occur more slowly when the dark period exceeded 16 h). The increased density of rhizobial cells near heterocysts was observed.

The coccoid shape of rhizobial cells under joint cultivation has been noticed. This phenomenon has been more than once emphasized in the literature as occurring under unfavorable conditions of RNB development. The change of rhizobial cell shape was reported also in the case of joint cultivation of *Rh. leguminosarum* from *Vicia faba* with the unicellular green alga *Chlamydomonas reinhardi* [20]. However, when *Rhizobium* cells were transferred to a selective medium (bean broth), the cocci transformed into typical mobile rods, 0.5–0.9 × 1.2–3.0 μm, with two to six peritrichous flagella or with a single polar or subpolar flagellum (Fig. 1b).

The affinity of *N. paludosum* to other species of rhizobia was studied in further experiments. *N. paludosum* was shown to have no specificity to a number of RNB species (*Rh. leguminosarum*, *Rh. galegae*, *Rh. trifolii*, and *Mesorhizobium loti*); moreover, RNB cells reproduced under conditions that were nonspecific for them (cyanobacterial slime). In further work, cultivation conditions, temperature, duration of illumination, and nutrient medium composition were adjusted in such a way as to bring the titer of rhizobia to 10^8–10^9 cells/ml of the medium. Generally, after the introduction of RNB into the nitrogen-free medium with *N. paludosum*, their numbers initially decreased, apparently due to the insufficient supply of CB exometabolites. During the cultivation, the mass of *N. paludosum* increased followed by an increase of the quantity of RNB cells (Fig. 2).

This pattern is confirmed by the previously obtained data on the efficiency of nonsimultaneous mixing of *N. paludosum* cells with the population of rhizobia (Fig. 3).

Taking into account the final result of mutual influence of microorganisms, the relationship between the partners can be classified as metabiotic, according to Nikitin [21].

The stability of a constructed association is determined by its ability to persist in time and to recover to the initial level after severe impact (Table 1).

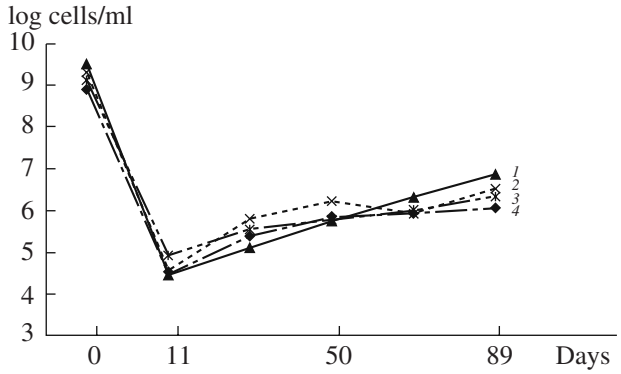

Fig. 2. Quantitative dynamics of the cells of *Rh. leguminosarum* (*1*), *Rh. trifoli* (*2*), *Mesorhizobium loti* (*3*), and *Rh. galegae* (*4*) in a binary composite with *N. paludosum.*

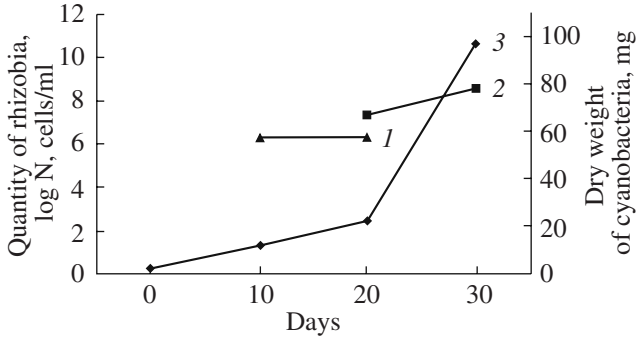

Fig. 3. The effect of *N. paludosum* on the quantity of *Rh. leguminosarum* cells at inoculation of *Rhizobium* on day 10 (*1*) and 20 (*2*); dry weight of cyanobacteria (*3*).

Table 1. Stability of cyano–rhizobial consortia in the course of time and under different maintenance conditions

Conditions	Period of observation, months	Revival of cyanobacteria*	Titer of rhizobia****	
			In the beginning of experiment	After exposure
Liquid nitrogen-free medium (transfers every 30–45 days)	18	15–18 day	10^7 cells/ml	10^5 cells/ml
Cyano-rhizobial mat, storage in air-dried state	2	10–15 day	10^6–10^7 cells/ml	1.4–2.0×10^6 cells/ml
Storage in sterilized soil in air-tight state**	1	15 day	0.9×10^5 cells/ml	1.0×10^5 cells/g soil
	6	15 day	0.9×10^5 cells/ml	1.7×10^4 cells/g soil
Storage of cyano-rhizobial lawn*** on soil in air-dried state	2	15 day	8.6×10^6 cells/ml	2.5×10^6 cells/g soil

* The beginning of revival was registered by appearance of hormogonia.

** Maintenance in air-dried state in air-tight plastic bags.

*** Cyano-rhizobial lawns were obtained by inoculation of the culture onto sterile soil (80 g) in petri dishes and growing in a luminostat for one month before drying.

**** The titer of inoculum (liquid medium), cell/ml, is given for the beginning of the experiment and the titer of rhizobia in the substrate, cells/g, is given after exposure.

Table 2. The effect of the cyano–rhizobial consortium and its partners on pea growth in water cultures, average per one plant

Variant	Root system volume, cm^3	Number of nodules	Above-ground part, % of the control	
			Height	Dry matter
Rh. leguminosarum	9.6	24.6	100	100
N. paludosum	8.8	–	100.3	110.3
N. paludosum + *Rh. leguminosarum*	13.4	31.7	122.0	121.9

Our observations suggest comparative stability of the association: the viability of CRC in liquid culture was tested 18 months after it had been formed. Different resistance of the partners to drying was noted. The survival of cyanobacteria is not surprising, because there are the well-known examples of their revival after storage for several years in herbarium, over sulfuric acid, and as a material capable of regrowth in soil [22]. CRC persisted at drying, but the cell titer of the rhizobial component in the population decreased with time in all cases.

The efficiency of artificial CRC as compared with the standard method of RNB application was tested on different cultures.

In water cultures, inoculation of pea by the new method resulted in the following noticeable consequences, which were particularly marked in the periods of budding and blooming. First of all, the growth of roots and the number and size of nodules increased as compared with inoculation with rhizobia alone (Fig. 4, Table 2). The color of the nodules changed: as a result of accretion with CRC, native gently pink nodules became "shaggy" and gained the typical color of CB (Fig. 5).

The topography of arrangement of cyanobacteria is of interest: they were absent on thin growing roots (Fig. 6).

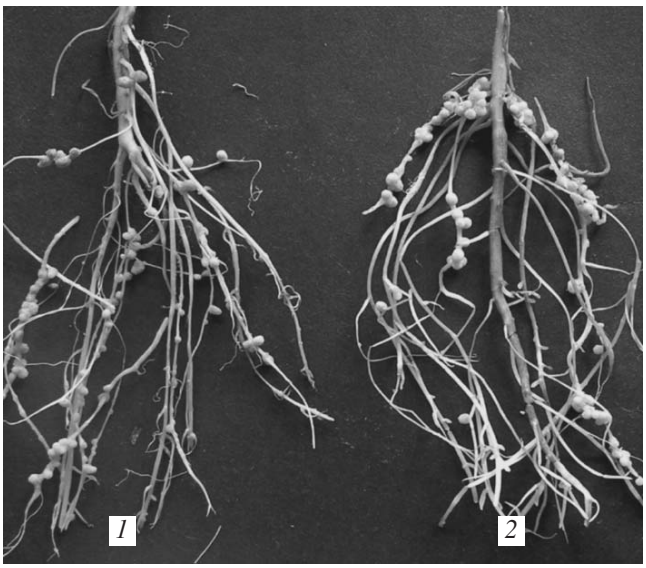

Fig. 4. Effect of inoculation of pea with *Rh. leguminosarum* (*1*) and *N. paludosum* + *Rh. leguminosarum* (*2*) on development of nodules.

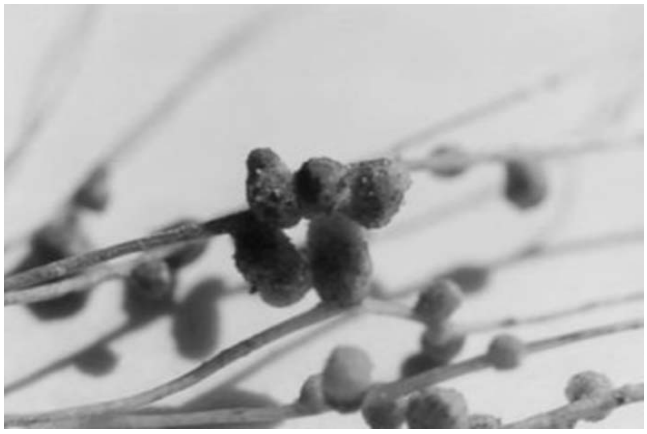

Fig. 5. Growth of a cyano–rhizobial consortium of pea nodules.

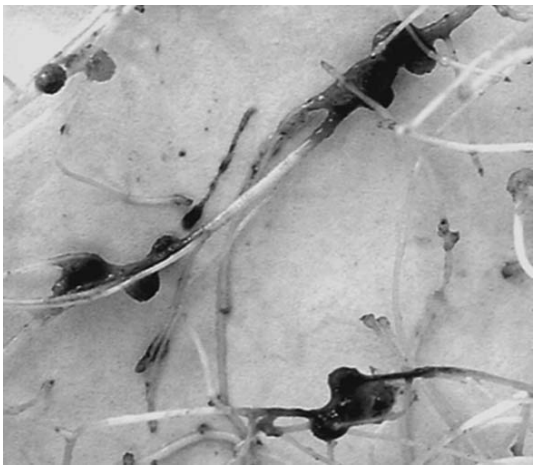

Fig. 6. Aggregation of a cyano–rhizobial consortium on pea nodules.

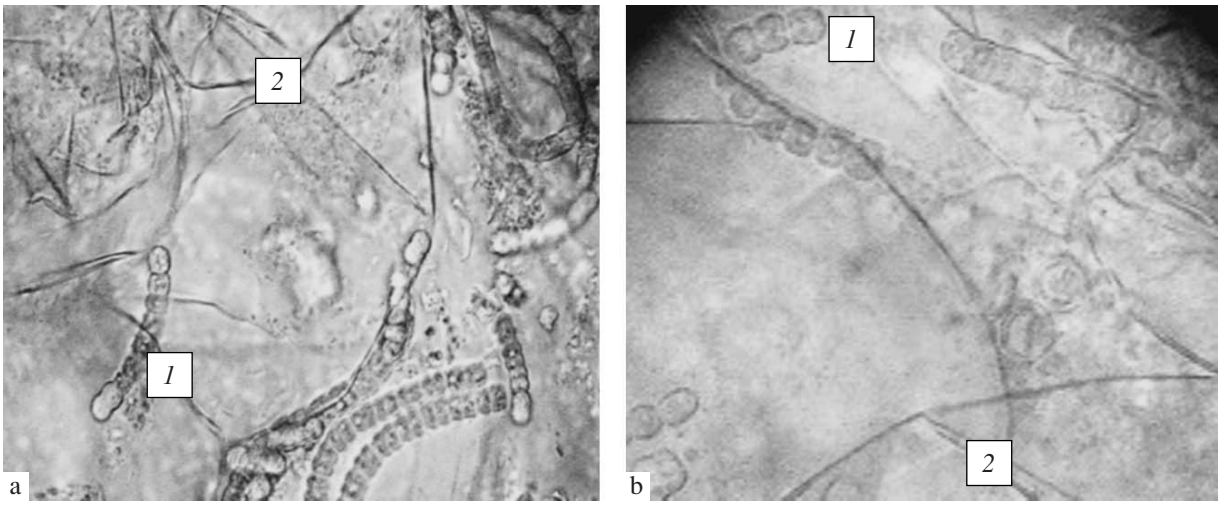

Fig. 7. Cyanobacteria in the cells of a pea nodule: a, at ×1350 magnification; b, at ×1350 magnification + digital magnification. *1*, *N. paludosum* filament; *2*, nodule tissue cell wall.

Dissection of the nodules showed that *Nostoc* hormogonia penetrated into the nodules and could be seen on the photograph as multicellular formations (Fig. 7).

The presence of CB in Egyptian clover was established for the first time by Venkataraman [23] and confirmed by Watanabe [24]. Such reports are sporadic and relate not to artificial formations, like in our case, but to natural phenomena. We have been the first to reveal the affinity of these organisms at formation of artificial consortia acting on garden peas. Further work on estimation of the duration of partners' coexistence in the nodules and the effectiveness of their activity is required. The roots of many plants are apparently also sensitive to CB inoculation; cyanobacteria are localized under root caps and in the hollows of stems in rice, tobacco, *Solanaceae*, and other plants [25].

Newly organized syntrophic systems can change the morphometric parameters of plants, similar to our case (Table 2).

It can be seen that CRC inoculation results in an increase of plant growth characteristics as compared with inoculation of plants by monocultures.

Another aspect of CRC impact, namely, on the nitrogen-fixing ability, was studied in clover. It was shown that the treatment of meadow clover seeds by CRC enhanced the activity of nitrogen fixation and, consequently, the amount of fixed atmospheric nitrogen (Table 3).

Thus, the results of laboratory studies of pea in water cultures, showing a positive effect on plant growth, were confirmed under soil conditions with meadow clover, the most widespread legume in the non-chernozem area.

Table 3. The effect of the cyano–rhizobial consortium on nitrogenase activity and coefficient of symbiotic nitrogen fixation by meadow clover plants, Kudesnik variety

Variant	Nitrogenase activity, μM of C_2H_4/g of roots h^{-1}	Coefficient of symbiotic nitrogen fixation, %
Control (without inoculation)	8.59 ± 0.24*	81.7 ± 0.8
Rh. trifolii	8.62 ± 0.19	86.1 ± 0.8
N. paludosum + *Rh. trifolii*	9.18 ± 0.16	90.7 ± 1.1

* $P < 0.05$.

The presented results show the prospects of arranging artificial consortia on the basis of a phototrophic cyanobacterial component. Previously we have shown that such consortia may include different physiologically active *Pseudomonas* strains, *Agrobacterium* and *Arthrobacter* species [14]. The developments based on the antifungal properties of cyanobacterial associations [26] and on their insecticidal effect [27] may be relevant. Advanced research is related, for example, to application of cyanobacterial consortia for addressing various pollutants. It has been shown, for example, that cyanobacterial associations can degrade oil pollutions [28].

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