ISSN 0026-2617, Microbiology, 2014, Vol. 83, No. 4, pp. 391–397. © Pleiades Publishing, Ltd., 2014.
Original Russian Text © B.K. Zayadan, D.N. Matorin, G.B. Baimakhanova, K. Bolathan, G.D. Oraz, A.K. Sadanov, 2014, published in Mikrobiologiya, 2014, Vol. 83, No. 4, pp. 467–474.

EXPERIMENTAL ARTICLES

Promising Microbial Consortia for Producing Biofertilizers for Rice Fields

B. K. Zayadan[a, 1], D. N. Matorin[b], G. B. Baimakhanova[a], K. Bolathan[a], G. D. Oraz[a], and A. K. Sadanov[c]

[a] *Al-Farabi Kazakh National University, Almaty, Kazakhstan*
[b] *Lomonosov Moscow State University, Moscow, Russia*
[c] *Institute of Microbiology and Virology SC MES RK, Almaty, Kazakhstan*

Received January 16, 2014

Abstract—Two cyanobacterial cultures from rice paddies of Kyzylorda Provence, Kazakhstan were isolated and characterized: *Anabaena variabilis* and *Nostoc calsicola*. Based on these cultures, new consortia of cyanobacteria, microalgae and *Azotobacter* were developed: ZOB-1 (*Anabaena variabilis*, *Chlorella vulgaris*, and *Azotobacter* sp.) and ZOB-2 (*Nostoc calsicola*, *Chlorella vulgaris*, and *Azotobacter* sp.). High growth rate and photosynthetic activity of microalgae were observed in these consortia. The active consortium ZOB-1 was selected, which improved germination and growth of rice plants. ZOB-1 was recommended as a biostimulator and biofertilizer for crops.

Keywords: consortium, cyanobacterium, *Azotobacter*, microalgae, biostimulation and biofertilizers

DOI: 10.1134/S0026261714040171

Cyanobacteria play an important role in enhancing soil fertility by fixing atmospheric nitrogen [1]. Application of nitrogen-fixing cyanobacteria in agriculture is promising due to their involvement in nitrogen turnover, which substantially affects the productivity of higher plants. Along with other soil organisms, cyanobacteria participate in formation of soil humic compounds. Many species of cyanobacteria used for soil algalization act also as fungistatics and fungicides [2]. A positive effect of cyanobacterial inoculation is due not only to their nitrogen-fixing activity, but also to production of biologically active substances and their stimulating influence on heterotrophic nitrogen-fixing microorganisms. In recent years, associations of microorganisms (consortia), which reveal the biotechnological potential of microorganisms more completely, are used in agricultural biotechnology, rather than single-species populations of microorganisms. This provides for multidirectional action and not only the additive but also the synergic effect; the chances for survival of the inoculum are increasing as well [3]. The noted advantages of microbial consortia over monocultures are the following: their universality (polyvalence); capacity for self-regulation by changing the relative abundance of species of the consortium; capacity for utilization of the substrates with heterogeneous composition; the possibility to use poorer and less valuable nutrient media due to their enrichment with the products of microbial metabolism; lower cost and better cost-effectiveness; and the possibility to develop efficient biological systems in numerous industries related to microbiology. Cyanobacteria as components of such associations have not been studied in detail. They are, however, an indispensable component of soil microbiota with a potential for mass development on its surface, capable of agronomically significant nitrogen fixation, and are the primary producers of organic matter. The ability to synthesize physiologically active substances stimulating root formation in higher plants makes them the subject of close attention of microbiologists and biotechnologists. However, from the standpoint of directional designing of microbial consortia, the unique communicability of cyanobacteria (i.e., their ability to establish long-term or temporary relations with the most ordinary soil bacteria) has remained outside the scope of scientific interest. All of the above determined the timeliness of the studies of these microorganisms as biotechnological subjects [4–6].

The goal of the present work was to isolate and study the cultures of nitrogen-fixing bacteria from rice paddies and to create microbial consortia on their basis for obtaining efficacious biological preparations with a view to using them in rice plant agrobiotechnology.

MATERIALS AND METHODS

The samples from the rice fields of the Zhakhaev Kazakh Research Institute of Rice Growing, Republic

[1] Corresponding author; e-mail: zbolatkhan@mail.ru

of Kazakhstan, were the subject of research. The material was collected from March to June 2012. The species composition of cyanobacteria in the samples from various aquatic ecosystems was determined according to Sirenko using different identification guides [7–9]. In order to isolate an algologically pure culture from the enrichment culture, microbiological methods of separation and subculturing were used. In order to assess the activity of cyanobacteria, their algologically and bacteriologically pure forms were used [10, 11].

To check for bacterial purity, the cultures were transferred into sterile 0.25% nutrient broth. The culture purity was determined by the broth turbidity. Microscopy was performed using a Meiji microscope (Japan). The growth dynamics of the cultures were studied by determining their optical density at 750 nm using a PD-303 spectrophotometer (Japan) [12].

Photosynthetic activity was measured using a Water PAM (Walz, Germany) pulse fluorometer. Constant (Fo) and maximal fluorescence (Fm) were recorded in darkness-adapted samples. The measurements of light relationships between the fluorescence parameters in the light were made by sequentially increasing light intensity from 0 to 800 μE/(m^2 s) [13]. The illumination time was 50 s. At the end of each illumination session, the Fm' parameters, as well as the fluorescence yield in the light F(t), were recorded at a certain intensity using the saturating flash 0.8 s, 3000 μE/(m^2 s). Based on all the parameters, the quantum yield of photochemical conversion of absorbed light energy in the photosystem 2 (PS2) as the ratio Y = (Fm' – Ft)/Fm' and the relative noncyclic electron transport rate (ETR) at a given light intensity were calculated. The electron transport rate was calculated according to the formula ETR = Y × E_i, ×0.5, where E_i is intensity of illumination, μE/(m^2 s) [13, 14].

In order to create the consortium, *Chlorella vulgaris* Z-1 strains from the collection of the Department of Biotechnology, Al-Farabi Kazakh National University, and *Azotobacter* sp. strain from the collection of the Institute of Microbiology and Virology, Republic of Kazakhstan, were used together with cyanobacteria. The algal cultures were cultivated in Gromov medium; the culture of *Azotobacter* sp. was grown in Ashby medium. The medium for cultivation was composed of these two media in equal portions.

The laboratory experiments on the germination of the Ak Marzhan rice seeds were carried out according to the method of Nurgasenov et al. [15]. The treated seeds were placed in petri dishes with moistened three-layer filter paper. The plates contained ten seeds each. The moist chambers were maintained under illumination at 20–22°C, with the number of germinated seeds and the sprout growth rate being determined daily.

All the measurements were made in at least five replicates. The figures show the results of three series of experiments in calculating the standard deviations for the probability $p > 0.95$.

RESULTS AND DISCUSSION

Construction of consortia for use in rice agrobiotechnology necessitates the isolation of highly productive valuable strains and species of cyanobacteria. These strains should be comparatively studied under certain cultivation conditions.

In the course of our studies, four algologically pure cultures of cyanobacteria (*Anabaena variabilis*, *Spirulina* sp. K-1, *Oscillatoria* sp. K-1, and *Nostoc calsicola*) were isolated from rice paddies. As a result of repeated transfers, the populations of algologically pure cyanobacteria developed from one cell were obtained.

Nostoc calsicola belongs to cyanobacteria, order *Nostocales*, genus *Nostoc*. The trichomes are single, straight, consist of spherical cells, and include heterocysts and, less frequently, akinetes. Gromov medium was used for cultivation.

Spirulina sp. K-1 beongs to cyanobacteria, order *Oscillatoriales*, genus *Spirulina*. This is a filamentous organism, with the cells forming regular spirals. The trichomeres are light, blue-green; their diameter is 1–2 μm; the pitch of the helix is 2.7–5 μm. They mainly grow on Zarrouk medium forming aggregates on the walls of the vial.

Oscillatoria sp. K-1 is a cyanobacterium of the order *Oscillatoriales*, genus *Oscillatoria*. The trichomes are bluish-green, straight, not interlaced at the transverse septa, not tapered at the ends; the length is more than the width. Isolated and cultivated on Gromov nutrient medium.

Anabaena variabilis is a cyanobacterium of the order *Nostocales*, genus *Anabaena*. The trichomes are single, very often in glomeruli, consist of spherical cells among which heterocysts and, less frequently, akinetes occur. Their trichomes structurally resemble those of *Nostoc*. They are usually curled up in a spiral or circular fashion, less frequently straight. Gromov medium is used for cultivation.

Determination of the ability of the isolated cyanobacterial strains to grow without an added nitrogen source. In order to reveal the capacity of cyanobacteria for nitrogen fixation, the dynamics of their growth as biomass increment on medium without a source of nitrogen are usually studied [16, 17]. The results of our experiments on cultivation of the cultures in nitrogen-free medium showed that, of all the cyanobacteria isolated, the cultures of *A. variabilis* and *N. calsicola* had the highest activity (Fig. 1). The growth of the cells of *Spirulina* sp. K-1 and *Oscillatoria* sp. K-1was by far lower compared to the growth of *A. variabilis* and *N. calsicola* cultures.

The high activity of growth of *A. variabilis* and *N. calsicola* cultures in the nutrient nitrogen-free

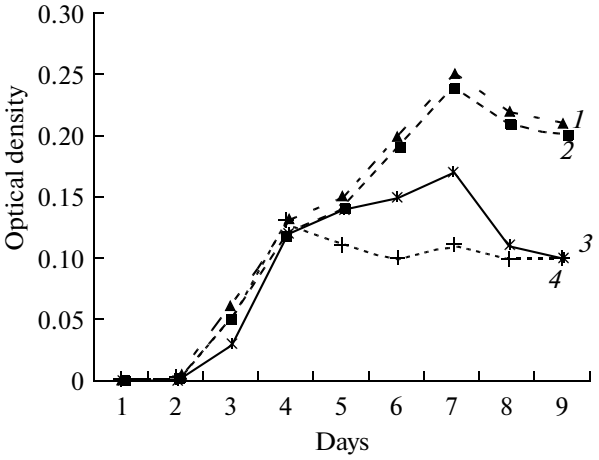

Fig. 1. Growth dynamics of the cyanobacterial cultures of *A. variabilis* (*1*), *N. calsicola* (*2*), *Oscillatoria* sp. K-1 (*3*), and *Spirulina* sp. K-1 (*4*) in the nitrogen-free medium.

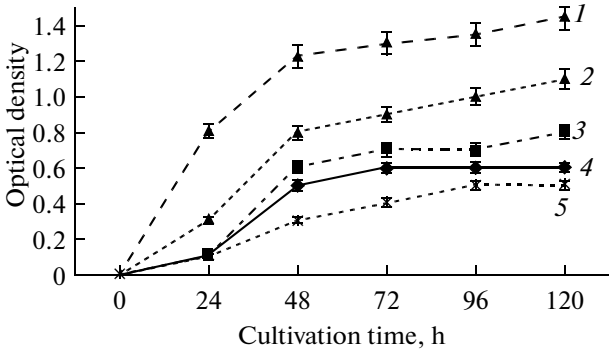

Fig. 2. Growth dynamics of *A. variabilis* cells at different medium pH values: pH 8 (*1*), pH 6 (*2*), pH 4 (*3*), pH 2 (*4*), and pH 10 (*5*).

medium indicated that these cultures obtained nitrogen more efficiently by binding it from the atmosphere. The strain of *A. variabilis* is known to have a high growth rate in nitrogen-free medium, which correlates with the high rate of heterocyst formation and a high nitrogenase activity [12].

Effect of pH on the growth of *A. variabilis* and *N. calsicola* cells. Optimum conditions are required for the cultivation of an active consortium. Cyanobacteria are exposed to a number of ecological factors, the most significant of which are pH, temperature, and illumination. In order to achieve high results during cultivation of cyanobacteria, pH must be optimal for their growth. Resistance response of the representatives of various taxa to changes in acidity varies. Growth of many cyanobacteria occurs in neutral or slightly alkaline medium with the optimal pH 7.2–7.5 [18]. Acidity of the medium affects the stability of the medium components, their availability to cyanobacteria, and especially the assimilability of the vitamins [18].

We studied the effect of pH on the growth of cyanobacterial cells of *A. variabilis* and *N. calsicola* in the 2–10 range. For the growth of *A. variabilis* strain, the optimum value was within pH 6–8 (Fig. 2). Similar results were obtained for *N. calsicola* culture (data not shown).

Microscopic investigations showed that at pH 2, complete degradation of the cell content occurred. When the pH values were in the 2–4 and 8–10 range, 50 to 60% of the cells changed their shape from ellipsoidal to spherical; cell walls were damaged; and the cytoplasm was discolored. At pH over 8, the number of nonmotile, destroyed cells sharply increased. In the 7–8 pH range, the cells retained normal morphology. In other words, the 7–8 pH range was the most optimal for growth of *A. variabilis* cells.

The optimal temperature and illumination conditions for growth of the cultures isolated from rice paddies were also determined. The optimal temperature values for cultivation of the cyanobacterial cultures of *A. variabilis* and *N. calsicola* were 25–30°C under constant illumination at 3000–4000 lx, which was consistent with the literature data [19].

Development of microbial consortia based on the cultures of cyanobacteria and *Azotobacter* isolated from rice paddies. Development of microbial consortia based on nitrogen-fixing cyanobacteria and nitrogen-fixing bacteria offers new promise in agrobiotechnology and may be used for ecological purposes given the need for stably working microbial associations [20]. Highly active cyanobacteria *A. variabilis* and *N. calsicola* were chosen for the consortia. In order to intensify the process of nitrogen fixation, the strain of *Azotobacter* sp. was added to the consortium. The microalga *Chl. vulgaris* Z-1 was added to the consortium in order to adjust the pH values. It was shown that the strains of *A. variabilis* and *N. calsicola* alkalinized the medium to pH 8, while in the *N. calsicola–Chl. vulgaris* and *A. variabilis–Chl. vulgaris* consortia, pH returned to neutral after three and four days, respectively.

As a result, new consortia of cyanobacteria, microalgae, and *Azotobacter* bacteria were developed: ZOB-1 (*A. variabilis, Chl. vulgaris, Azotobacter* sp.) and ZBOB-2 (*N. calsicola, Chl. vulgaris, Azotobacter* sp.), which exhibited good growth. Figures 3 and 4 show the dynamics of growth of two consortia with and without *Azotobacter* sp. upon cultivation for eight days. It may be seen that both consortia consisting of three microorganisms exhibited better growth than the consortia without *Azotobacter* sp., presumably owing to the fact that the strain *Azotobacter* sp. increased nitrogen fixation in the consortium.

Contrary to the growth of pure cultures (Fig. 1), a decline in growth by days 7–8 was not observed in the consortia created, probably because more active dinitrogen fixation proceeded in the consortia and micro-

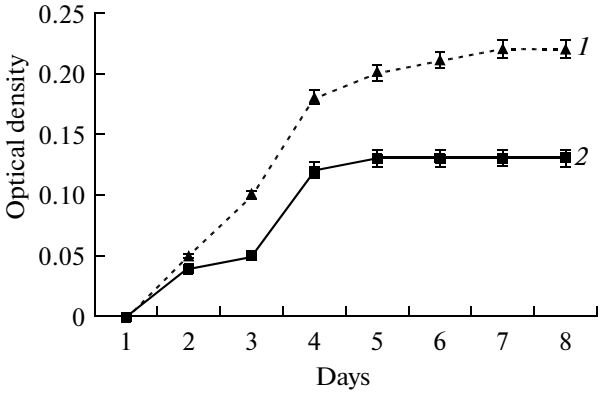

Fig. 3. Growth dynamics of the *N. calsicola–Chl. vulgaris–Azotobacter* sp. (*1*) and *N. calsicola–Chl. vulgaris* (*2*) consortia.

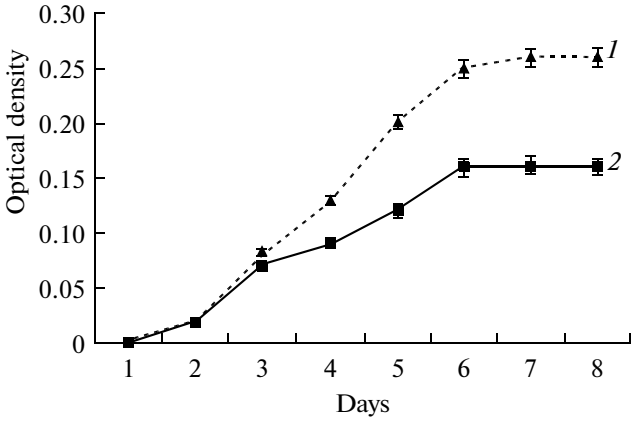

Fig. 4. Growth dynamics of the *A. variabilis–Chl. vulgaris–Azotobacter* sp. (*1*) and *A. variabilis–Chl. vulgaris* (*2*) consortia.

organisms did not experience the depletion of this element upon long-term cultivation.

Microscopic study of the morphology of the consortia was carried out. Figure 5 shows the microphotographs of bacteria in the consortia ZOB-1 and ZBOB-2. The filamentous cyanobacteria *A. variabilis* and *N. calsicola* (without staining) had light blue-green trichomes; their diameter was 1–2 μm; the pitch of the helix is 2.7–5 μm. *Chl. vulgaris*, a unicellular microalga, was represented by spherical cells 2 to 10 μm in size. For *Azotobacter* sp. cells to be observed (Figs. 5c, 5d), the cells were stained with gentian violet solution. The *Azotobacter* sp., a gram-negative bacterium, was represented by single or paired rods with rounded ends. The rod length varied between 2 and 3 μm.

Photosynthesis of cyanobacteria and the cyanobacteria-based consortia. High photosynthetic activity of cyanobacteria in the ZOB-1 and ZBOB-2 consortia was confirmed when the photosynthesis was studied using the fluorescence methods. Figure 6 shows the light curves of the relative noncyclic electron transport rate (rETR) in the pure cultures of *A. variabilis* and *N. calsicola* and in the consortia ZBO-1 and ZBOB-2 developed on their basis. Upon long-term cultivation in nitrogen-free media, the noncyclic transport rate associated with oxygen release and CO_2 fixation sharply increased in the cyanobacteria within the ZOB-1 and ZBOB-2 consortia. This resulted from probably better conditions for dinitrogen fixation in these consortia. Thus, no nitrogen starvation occurred during long-term cultivation of the consortia in nitrogen-free media. The relationship between the photosynthesis of microalgae and nitrogen content in the medium was demonstrated by us earlier in [21, 22].

The study of the effect of consortia on the germination of rice seeds and growth of rice sprouts. In order to reveal the agrobiotechnological potential of cyanobacterial consortia, it was necessary to conduct in situ studies because the features of the introduced microbial populations might not manifest themselves under these conditions. Loss of functionality, which is a charge for survival in the composition of a natural microbial community, is not infrequent. The use of a consortium is known to enhance the process of nitrogen fixation [23–25]. It was also noted that the stimulating effect of cyanobacteria on plants was associated with their disinfecting action.

The consortia were tested according to the parameters of their influence on the germination of rice seeds and the growth of rice sprouts. The seeds were treated with a suspension of two consortia composed of *A. variabilis, Chl. vulgaris, Azotobacter* sp. and *A. variabilis, Chl. vulgaris.* Figure 7 shows the photographs of rice sprouts five days after treatment of the seeds with the consortium preparations. As seen from the figure, the rice seeds treated with *A. variabilis, Chl. vulgaris*, and *Azotobacter* sp. showed the highest degree of germination and an increase in the length of the sprouts. In other words, treating the seeds with the consortium suspensions improved seed germination compared to the control. It is possible that the growth characteristics of rice plants might have improved owing to an increase in nitrogen-fixing activity of the consortia.

The table shows the quantitative parameters of the influence of ZOB-1 with and without *Azotobacter* sp. on the germination and growth of the Ak Marzhan variety of rice. The treatment of seeds with the consortium cell suspensions exerted a positive effect on the growth of rice cultures. Analysis of the number of rice plants and stem length revealed that the germination of the seeds treated with the consortium ZOB-1 without *Azotobacter* sp. was 90% and the plant height increased by 19.2%; in the case of ZOB-1 with *Azotobacter* sp., these parameters were 96 and 27%, respectively, compared to the control (table). The results of laboratory experiments showed that treatment of rice seeds by a suspension of the *A. variabilis, Chl. vulgaris,*

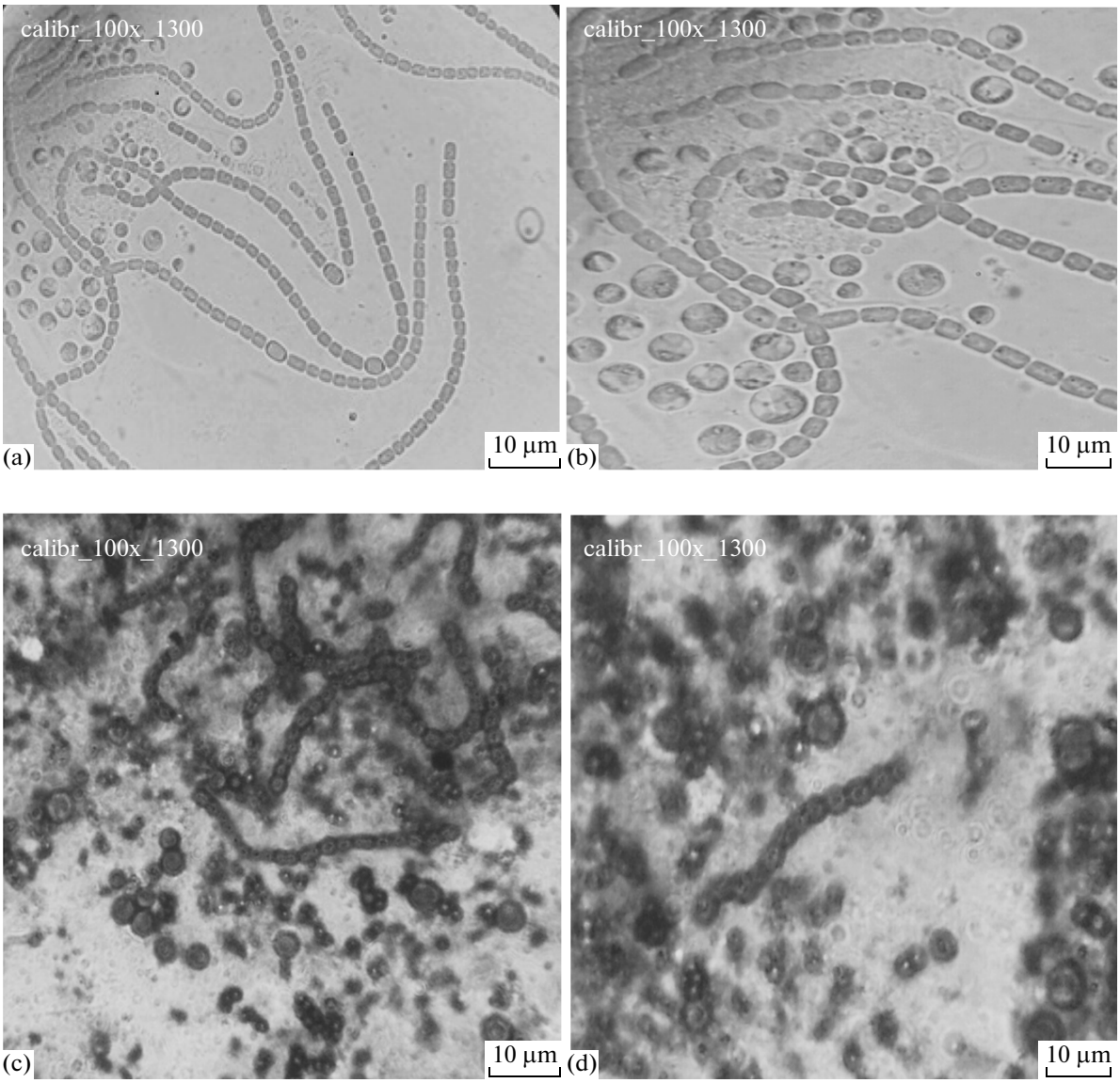

Fig. 5. Culture morphology in artificial consortia without staining (a, b) and after staining with the dye gentian violet (c, d). Designations: (a, c) ZBOB-2 (*N. calsicola*–*Chl. vulgaris*–*Azotobacter* sp.); (b, d) ZOB-1 (*A. variabilis*–*Chl. vulgaris*–*Azotobacter* sp.).

Effect of the consortium ZOB-1 with and without *Azotobacter* sp. on the germination of rice seeds and the growth of rice plants

Samples	Number of germinated seeds, %	Length of the rice plant sprouts (cm) and % of the control
Control seeds	80	13 ± 0.3 100%
Seeds treated with suspension of the ZOB-1 consortium without *Azotobacter* sp.	90	15.5 ± 0.3 119.2%
Seeds treated with suspension of the ZOB-1 consortium with *Azotobacter* sp.	96	16.5 ± 0.5 127%

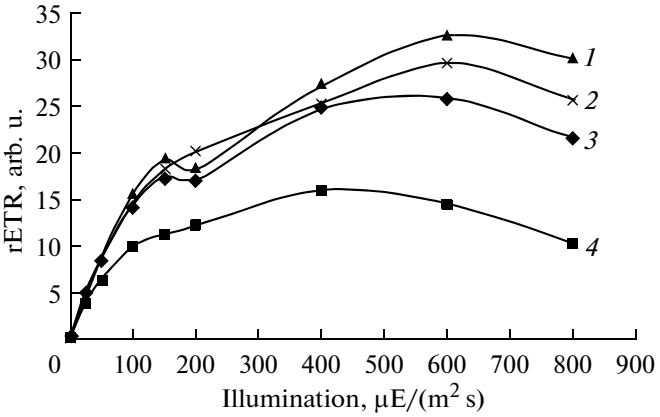

Fig. 6. Light curves of the relative noncyclic electron transport rate (rETR) in the pure cultures of the cyanobacteria *N. calsicola* (*4*) and *A. variabilis* (*3*) and in the consortia ZOB-1 (*2*) and ZBOB-2 (*1*) during long-term (7 days) cultivation in nitrogen-free medium.

and *Azotobacter* sp. consortium exerted the greatest stimulating effect on the germination, stem length, and development of the plants. The stimulating effect was also noted for ZBOB-2, albeit to a lesser degree.

Thus, two microbial consortia were developed using the cyanobacterial strains isolated from rice paddies: ZOB-1 (*A. variabilis–Chl. vulgaris–Azotobacter* sp.) and ZBOB-2 (*N. calsicola–Chl. vulgaris–Azotobacter* sp.), which may be proposed for use in the agrobiotechnology of rice crops with a view to improving the germination of plants and enriching soil with fixed nitrogen. Their advantages include resistance of the cyanobacterial partner to extreme conditions, extraordinary ecological valence, rapid growth rates, autonomy from the environmental content of very scarce carbon and nitrogen compounds, and the possibility of the synthesis of growth-stimulating substances.

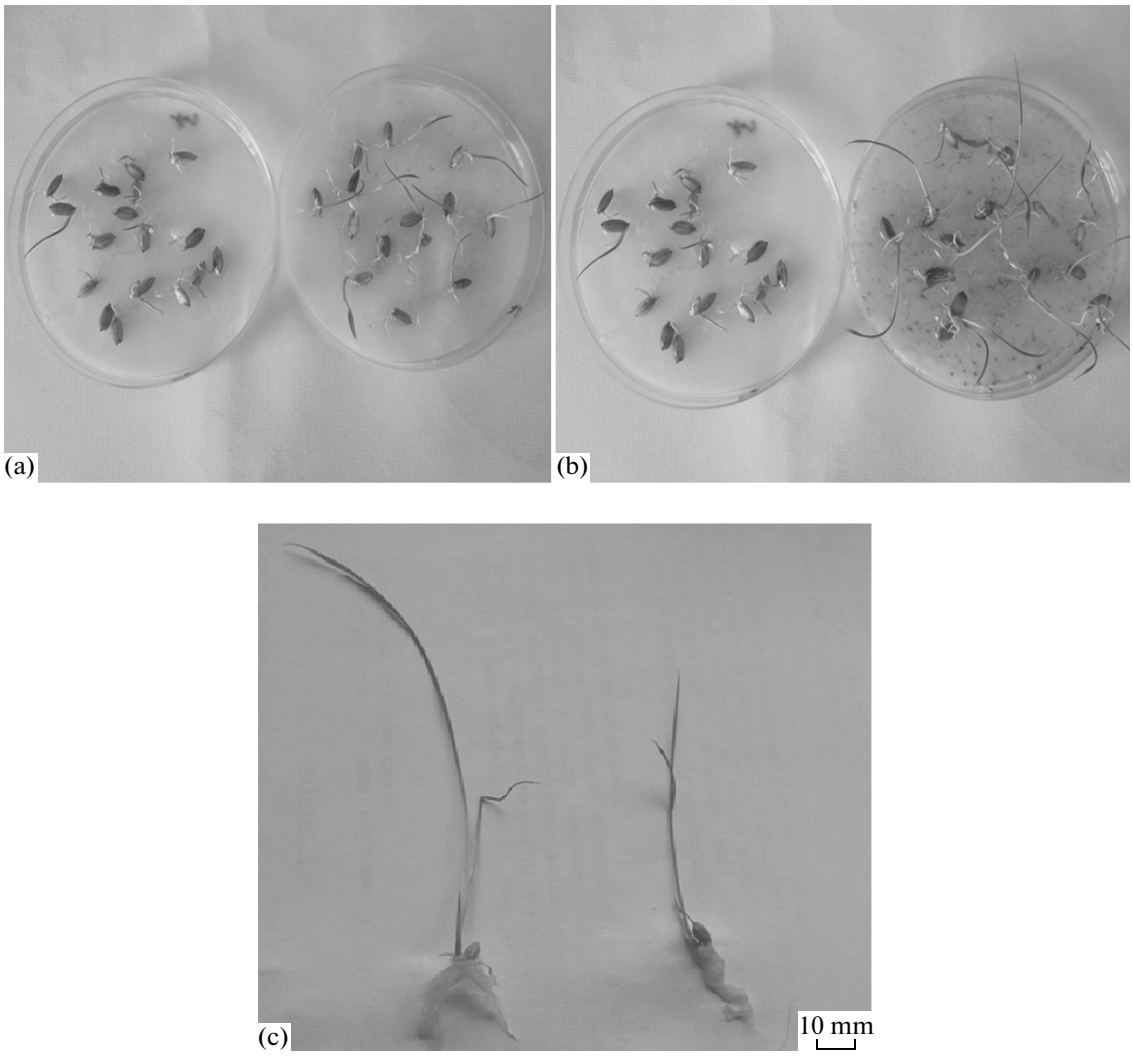

Fig. 7. Photographs of rice sprouts (a, b) 5 days after treatment of the rice seeds of the variety Ak Marzhan with the preparations of different consortia: (a) *A. variabilis–Chl. vulgaris*, (b) ZOB-1 (*A. variabilis, Chl. vulgaris, Azotobacter* sp.). Left dishes: the control, untreated seeds. The lower photograph (c): plant sprouts 10 days after cultivation; on the left: the plant from the seeds treated with ZOB-1; on the right: the plant from untreated seeds, the control.

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