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OPTIMIZATION OF THE SEARCH FOR HIGHLY EFFICIENT NODULE BACTERIA STRAINS FOR INOCULATION OF LEGUME PLANTS

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Abstract

Highly effective strains of nodule bacteria are the main component of microfertilizers used in the cultivation of leguminous plants. Using artificial inoculation with rhizobia, it is possible to achieve a significant increase in the yield of this crop. The most common way to search for highly effective strains of these microorganisms is to obtain them from the nodules of the plants themselves with subsequent selection of strains that most effectively affect plant yield. In this work, we analyzed bacteria obtained from nodules collected at different stages of development of such leguminous plants as *Trifolium hybridum* L., *Lotus corniculatus* L., and *Galega orientalis* Lam. It is shown that each plant enters into symbiosis with only one specific type of bacteria, but with a large number of its genetic variants, each of which can potentially have individual symbiotic characteristics. The greatest variety of strains was found in the nodules of *Trifolium hybridum*, and the microsymbionts of *Galega orientalis* were characterized by uniformity. It was found that at the beginning of the development of all three plant species, the nodules are formed by the most diverse strains of rhizobia which have high nitrogen-fixing activity ranging from 3.17 to 32.70 $N_2 \cdot plant^{-1} \cdot h^{-1}$ for microsymbionts of the *L. corniculatus*, from 10.08 to 30.75 $N_2 \cdot plant^{-1} \cdot h^{-1}$ for microsymbionts of *G. orientalis*, from 9.91 to 32.42 $N_2 \cdot plant^{-1} \cdot h^{-1}$ for microsymbionts of *T. hybridum*. Among them, highly effective strains are found more often than in other periods. In the middle of the growing season, bacteria that form nodules on the roots of the studied leguminous plants have relatively leveled values of nitrogen fixation efficiency, from 6.35 to 19.13 $N_2 \cdot plant^{-1} \cdot h^{-1}$ for microsymbionts of the *L. corniculatus*, from 15.51 to 23.47 $N_2 \cdot plant^{-1} \cdot h^{-1}$ for microsymbionts *G. orientalis*, from 11.61 to 20.53 $N_2 \cdot plant^{-1} \cdot h^{-1}$ for microsymbionts of *T. hybridum*, and their activity of nitrogen fixation are lower compared to maximum activity found in strains from nodules at the beginning of the growing season. At the end of the life cycle, nodules on the roots of the analyzed plants are formed by microorganisms that fix nitrogen with different efficiency but are still inferior in this trait to the most active «spring» strains. Thus, we believe that the search for effective strains of nodule bacteria is most productive at the initial stage of plant development.

Keywords: rhizobia, leguminous plants, *Lotus corniculatus* L., *Trifolium hybridum* L., *Galega orientalis* Lam., stage of vegetation, nitrogen fixation, highly effective strains

Artificial inoculation with nodule bacteria (rhizobia) when growing legumes leads to a significant increase in the yield. For the first time, rhizobia from the root nodules of legumes were isolated and cultured by the Dutch scientist Martinus Willem Beijerinck (03/16/1851-01/01/1931) in 1888, and the first rhizobia-based bioproduct was manufactured in Germany in 1896 under the trade name Nitragin. In the USSR, the first batches of this fertilizer were produced in

1930-1935 [1]. Since then, various bioproducts, based on strains of nodule bacteria, have been used to grow many leguminous plants. Since rhizobia are specific to the host plant, each drug can only be used on a specific crop, depending on the strain of bacteria it contains [2-4].

To produce preparations of root nodule bacteria, it is first necessary to isolate and select a production strain corresponding to a legume plant of a certain species. Usually, bacteria are isolated directly from the nodule. A small amount of plant tissue is removed from a large pink nodule, stained with leghemoglobin, and rubbed with a spatula over the agar surface in a Petri dish. After certain selection procedures, the resulting strain can be used for pre-sowing inoculation of a crop.

Since the discovery of nodule bacteria, large-scale studies have been carried out on the principles and genetic mechanisms of the legume-rhizobium symbiosis emergence and functioning, and the components involved in this process from both the plant and bacteria have been identified and described [5-8]. Thus, it is now known that numerous bacterial genes, designated as *sym* genes, are responsible for the formation of symbiosis with a plant. The products of these genes are involved in microbe-plant interaction at different stages of the symbiosis formation. A complex sytem of *sym* genes includes the *nif* genes for synthesis and regulation of the nitrogenase enzyme responsible for nitrogen fixation, the *nod* genes encoding Nod factors (NF) that are responsible for the initiation and specificity of the symbiosis, and *fix* genes for nitrogen fixation that are often linked but not homologous to *nif* genes [9, 10].

It is known that rhizobia have high genome plasticity due to their recombination activity and involvement in intensive horizontal gene transfer. As a result, a large number of different strains of nodule bacteria with different symbiotic activities inhabit in the plant rhizosphere [11]. In a temperate climate zone, where there is a change of seasons, leguminous plants begin each life cycles by selecting their microsymbionts. In the wild, this choice is subject to a large number of different factors, i.e., edaphic, climatic, biotic and abiotic. A mutualistic interaction is formed the effectiveness of which is commensurate with the level of the plant's ability to ensure nitrogen fixation in the nodules. As a result, the plant can compete with other organisms and leave offspring. Ultimately, this leads to symbiotic interactions of varying effectiveness and, accordingly, to interaction with nodule bacteria that have different symbiotic properties.

In wild leguminous plants, symbiosis occurs with strains of rhizobia that have different nitrogen-fixing activities. In crop production aimed at high yields, the most effective nodule bacteria are required, capable of maximizing the supply of fixed nitrogen to the plant. Consequently, there is a need to select from a large number of bacteria nodulating the leguminous plant roots those strains that would be most effective and, with pre-sowing treatment in high concentrations, would ensure the formation of nitrogen-fixing nodules produced predominantly by these strains but not by native rhizobia. The problem remains relevant to this day, since new conditions for growing plants are emerging and new varieties are created that may react differently to bacterial strains previously used for inoculation.

In this work, by comparing the nitrogen-fixing activity of nodules at different stages of plant development in three legume species, we revealed its differences. On this basis, the assumption was formulated for the first time that the search for strains of nodule bacteria with the greatest nitrogen-fixing capabilities is more expedient at the initial stage of plant development.

The purpose of the work was to find out in what period of the plant growing season the search for highly effective strains of nodule bacteria will be the most productive.

Materials and methods. Symbiotic bacteria were isolated from the nodules

of *Lotus corniculatus* L., *Trifoliut hybridum* L. and *Galega orientalis* Lam. plants, 30 isolates from each plant. Nodules were collected at different stages of the host plant's growing season, i.e., in spring during bud swelling, April-May, in summer during bud formation, June, and in autumn after the plants set buds for the winter with the first frosts. A puncture of nodule tissue from the zone of microbial reproduction was made to isolate individual colonies on YM nutrient medium (0.1% yeast extract, 1% mannitol, 0.05% K_2HPO_4, 0.05% $MgSO_4$, 0.01 NaCl, 1.5% agar) [12]. One isolate from each nodule was studied.

Bacterial DNA was isolated by lysing cells in 1% Triton X-100 and 1% Chelex 100 suspension (Bio-Rad, USA). A small amount of bacterial mass was placed in tubes with 100 μl of 1% Triton X-100 and 1% Chelex 100 suspension, carefully resuspended and incubated at 95 °C for 10 min. The suspension was centrifuged at 12000 g for 3 min. The supernatant was used as a template for PCR.

The genetic diversity of the strains was assessed using RAPD analysis (random amplified polymorphic DNA) [13] with random primers LMBD 5′-gggcgctg-3′, AFK1 5′-caggcccatc-3′ and OPA 5′-gcgtccatc-3′ (an iCycler device, Bio-Rad, USA). The protocol was as follows: denaturation at 95 °C for 30 s, primer annealing at 33 °C for 40 s, elongation at 72 °C for 1 min 20 s, 35 cycles.

For PCR-RFLP analysis (restriction fragment length polymorphism) [14] of the 16S rRNA gene, small-cut restriction endonucleases Kzo91 and HaeIII were used. The 16S rRNA gene was amplified with universal primers fD1 5′-cccgg-gatccaagcttaaggaggtgatccagcc-3′, rD1 5′-ccgaattcgtcgacaacagagtttgatcctggctcag-3′, flanking a gene fragment of ~ 1500 bp [15]. To amplify the *recA* gene, primers RecAF 5′-ggcagttcggcaagggctcgat-3′ and RecAR 5′-atctggttgatgaagatcaccat-3′ were used [16].

Nucleotide sequences were determined (an Applied Biosystems 3500 automatic sequencer, Applied Biosystems, Inc., USA) using Big Dye Terminator v.3.1 kits (Thermo Fisher Scientific, USA) in accordance with the manufacturer's protocol. Analysis of nucleotide sequences was carried out using the LaserGene software package (DNAStar, Inc., USA). Nucleotide sequences for comparative analysis were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov).

Phylogenetic analysis of the studied strains was carried out based on multiple alignment (ClustalW) of sequenced fragments of the 16S rRNA and *recA* genes. Phylogenetic trees were constructed by the neighbor-joining method (NEIGHBOR) in the Megalign program from the LaserGene software package. Statistical significance of branching (bootstrap analysis) was assessed using the appropriate function of the Megalign program based on 1000 alternative trees.

The nitrogen-fixing efficiency of nodule bacteria was measured by the acetylene reduction method [17]. The roots of each inoculated plant were separated from the shoot, washed with sterile water and placed individually in 15 ml glass vial. The vials were closed with rubber stoppers, and acetylene (10% by volume) was added. Vials with samples and controls (with and without acetylene) were placed in a dark place. After 1 h of incubation, the concentrations of acetylene and ethylene were measured according to the standard protocol base on the time of each gas release (a gas chromatograph with a flame ionization detector GC-2014, Shimadzu, Japan). Acetylene and ethylene concentrations were calculated using the calibration curve. The nitrogen-fixing activity was calculated by the formula: $X = C_e/3$, where C_e is the concentration of ethylene in the test sample, 3 is the conversion factor from acetylene reduction to nitrogen fixation.

Each strain was analyzed in three biological replicates. Means (*M*) and standard errors of means (±SEM) were calculated.

Results. Soil under natural conditions contains a wide variety of rhizobia strains with different symbiotic efficiency. A legume plant, when germinating from

a seed or after re-growth in the spring of a perennial species, begins to interact with nodule bacteria. This leads to the formation of nitrogen-fixing nodules in which the further fixation of atmospheric nitrogen directly occurs [2, 18, 19]. This interaction is subject to a genetically programmed process on both the plant and bacterial sides (*nod* genes), during which legumes and microorganisms exchange biochemical signals. The latter ultimately trigger bacterial penetration into the root and the formation of a nodule structure [20-22]. However, the plant cannot evaluate the nitrogen-fixing activity of the strain, since the *nif* genes are responsible for it (to a greater extent) together with other rhizobial genes, which in one way or another affect the nitrogen fixation.

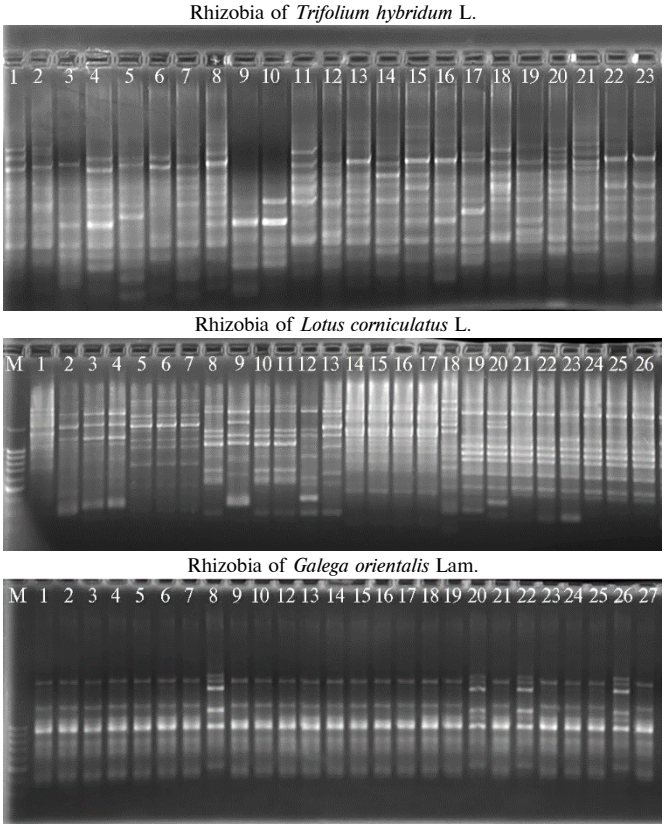

Fig. 1. Examples of electropherograms of RAPD profiles of bacterial isolates obtained from legume plant nodules at the beginning of the growing season. Isolate Nos, are indicated. M — 100 bp DNA length marker (JSC Evrogen, Russia).

Thus, leguminous plants can enter into symbiosis with strains of different effectiveness, and the formation of nodules will occur throughout the growing season, but with different intensity. In order to understand at what point in their development legumes interact with the most actively nitrogen-fixing strains of rhizobia, we collected nodules from the roots of *L. corniculatus*, *T. hybridum* and *Galega orientalis* at different stages of growing. Pure cultures of nodule bacteria were isolated from nodules collected from the roots of plants growing at the same site.

Analysis of genetic polymorphism using the RAPD method showed that bacteria forming nodules on the roots of the studied plants have different degrees of polymorphism depending on the host plant. The bacteria of hybrid clover were the most heterogeneous, and the least heterogeneous were those of eastern goat's rue (Fig. 1). This pattern was observed both in strains isolated from spring nodules and in strains from nodules collected at next stages of plant development.

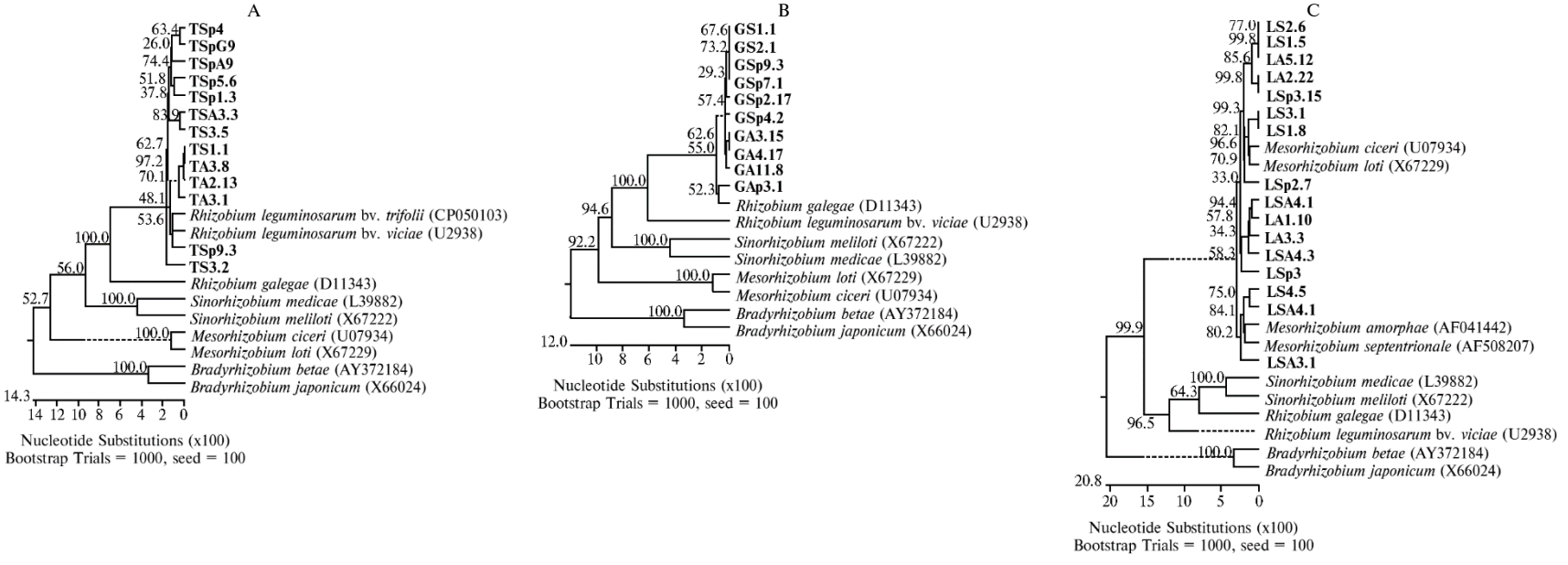

Fig. 2. Phylogenetic analysis of nodule bacteria in the studied legume species based on the 16S rRNA gene sequences: A — *Trifolium hybridum* L., B — *Galega orientalis* Lam., C — *Lotus corniculatus* L. The strains of microorganisms studied in this work are marked in bold.

In order to determine the species composition of bacteria, we conducted a PCR-RFLP analysis which identified phylogenetically homogeneous groups of microorganisms. Next, the 16S rRNA and *recA* genes were sequenced in the memebers of the groups.

By a comparative analysis of certain nucleotide sequences with similar sequences from the GenBank database, we identified the phylogenetic position of the studied strains (Fig. 2). It was found that all isolated strains of rhizobia from the nodules of *L. corniculatus* belong to the genus *Mesorhizobium* according to the sequence of both the 16S rRNA gene and the *recA* gene. Bacteria from the nodules of hybrid clover belong to the species *Rhizobium leguminosarum*, and microorganisms from the nodules of the oriental goat's rue belong to the species *Neorhizobium galegae*.

The nucleotide sequences of the studied strains are registered in the GenBank database under the following numbers:

16S rRNA gene: ON810504, ON819719, ON892022, ON834569-ON834571, OQ443231, OQ281287, OQ281289, OQ281290, OQ291567-OQ291569, OQ443237, OQ443238, OQ443243 OL872741, OL884458, OL884630, OL884456, OL884437, OM914882, OM914885, OM914886, OL884438, OL884442, OL884446;

recA: ON932443, ON932444, ON932446, ON932448-ON932450, OP899408, OQ102258, OQ102259, OQ102262-OQ102265, OQ111928-OQ111930, OM752310, OM811972, OM811974, OM811975, OM888662, OM888663, OM913901, OM913902, OM942752-OM942754.

Nitrogen-fixing efficiency of rhizobia strains from nodules collected at different stages of the host plant vegetation ($N_2 \cdot plant^{-1} \cdot h^{-1}$, average of 3 replicates, $M \pm SEM$)

Spring		Summer		Autumn	
strain	nitrogen-fixing efficiency	strain	nitrogen-fixing efficiency	strain	nitrogen-fixing efficiency
Rhizobia of *Lotus corniculatus* L.					
LSp1	3.17±0.61	LS1.5	10.21±0.60	LA1.10	4.01±0.34
LSp2.7	19.10±0.92	LS1.8	9.18±0.83	LA1.12	7.13±0.45
LSp3	32.70±1.71[a]	LS2.1	10.49±0.82	LA2.22	6.62±0.62
LSp3.8	12.24±0.34	LS2.6	19.13±0.88	LA3.3	15.19±0.75
LSp3.10	11.53±0.81	LSA3.1	8.15±0.45	LA5.2	16.30±0.37
LSp3.15	23.97±0.98	LS3.3	8.42±0.76	LA5.7	6.64±0.85
LSp4.1	10.85±0.23	LSA4.1	9.56±0.65	LA5.12	10.12±0.77
LSp4.3	9.34±0.46	LSA4.3	8.34±0.73	LA6	5.54±0.65
LSp4.8	10.34±0.34	LS4.4	6.35±0.43	LA6.3	4.99±0.46
LSp4.11	7.85±0.45	LS4.5	7.06±0.54	LA6.4	5.54±0.53
Rhizobia of *Galega orientalis* Lam.					
GSp1.5	15.03±0.89	GS1	23.47±0.98	GA3.11	23.21±1.34
GSp2.17	29.56±1.21	GS1.1	15.51±0.76	GA3.15	20.35±1.23
GSp3.1	24.31±1.01	GS1.6	19.13±0.56	GA4.3	21.01±1.54
GSp3.3	19.27±0.78	GS2.1	21.45±0.87	GA4.9	20.99±1.02
GSp4.2	10.09±0.65	GS2.8	17.17±0.65	GA4.17	25.39±0.98
GSp7.1	26.76±1.34	GS3	17.20±0.98	GA6.2	20.41±0.67
GSp7.4	11.42±0.76	GS3.3	19.23±1.03	GA7	22.13±1.06
GSp9.3	17.65±0.98	GS3.4	16.40±0.78	GA9.5	19.45±0.89
GSp9.7	30.75±1.34[a]	GS3.7	16.67±1.13	GA11.8	18.81±0.77
GSp9.9	12.78±0.24	GS3.8	18.11±0.99	GA11.9	20.71±1.23
Rhizobia of *Trifolium hybridum* L.					
TSpA4	25.17±0.34	TS1.1	20.53±1.08	TA1.2	25.61±1.01
TSp1.3	12.26±0.98	TS2.2	16.70±0.98	TA1.7	16.76±0.99
TSpA9	13.42±1.06	TS2.3	17.51±0.79	TA2.3	12.92±1.03
TSp2.4	32.42±1.90[a]	TS3	13.73±0.56	TA2.5	19.24±0.67
TSp3.1	28.61±1.05	TS3.2	15.38±0.89	TA2.13	16.16±0.97
TSp4	27.18±1.35	TS3.3	13.20±0.67	TA3.1	14.56±0.89
TSp5.6	30.24±0.98	TS3.4	14.51±0.89	TA3.2	20.02±1.22
TSpV1	12.48±0.56	TS3.5	12.34±0.78	TA3.6	12.31±0.95
TSpG9	14.27±0.78	TS4.1	13.91±0.79	TA3.8	11.56±0.68
TSp9.3	9.91±0.52	TS4.2	11.61±0.98	TA3.9	12.46±0.78

Note. The letter [a] marks the highest values.

To identify differences in the efficiency of nodule bacteria strains, we compared their nitrogen-fixing activity (Table). The analysis involved 10 strains for

each plant species, which were isolated from nodules collected during each period of development. It was found that, on average, the nitrogen-fixing activity of isolates from spring nodules is higher compared to summer isolates (see Table). This trend was found when analyzing rhizobia in all studied plants. By the middle of the growing season, the nitrogen-fixing activity of nodule bacteria decreases, and for microorganisms that form autumn nodules, it varies depending on the host plant.

Thus, the nitrogen-fixing efficiency of the bacteria from the *L. corniculatus* spring nodules averages 14.11 μg $N_2 \cdot plant^{-1} \cdot h^{-1}$, from summer nodules 9.59 μg $N_2 \cdot plant^{-1} \cdot h^{-1}$, and from autumn nodules 98.19 μg $N_2 \cdot plant^{-1} \cdot h^{-1}$. For rhizobia of hybrid clover, these figures are 20.61, 14.94 and 16.16 μg $N_2 \cdot plant^{-1} \cdot h^{-1}$, respectively, for eastern goat's rue nodules 19.76, 18.40 and 21.24 μg $N_2 \cdot plant^{-1} \cdot h^{-1}$.

When considering isolates from spring, summer and autumn nodules separately, an interesting pattern is revealed. Bacteria from spring nodules are characterized by a high variation in nitrogen-fixing activity. Thus, in the *L. corniculatus*, the upper and lower values average 37.7 and 3.17 μg $N_2 \cdot plant^{-1} \cdot h^{-1}$, respectively, while microorganisms from summer nodules demonstrate more equal values (see Table). The same turned out to be characteristic of rhizobia in other studied planr species. However, isolates from autumn nodules begin to differ in these parameters. It is obvious that plants, when interacting with a significant variety of strains of nodule bacteria at the beginning of the growing season, realize the chance to select for themselves the most suitable microorganisms.

The use of rhizobia to improve legume yields remains a priority for biological science. Some researchers emphasize that the use of local *Rhizobium* strains is preferable because they are better adapted to environmental changes [23]. The ability of native strains to positively interact with resident soil microbiota and their adaptability to local agroecological climatic conditions often explain their superiority over exotic commercial strains [24, 25]. It is known that the effectiveness of rhizobia strains depends on the plant species and on variety. Therefore, for each variety, ideally it is necessary to select its own strains of nodule bacteria [26]. In this regard, the problem of finding highly effective strains of rhizobia will not lose its relevance in the foreseeable future.

Today, one of the most common ways to search for highly effective strains of nodule bacteria is to select the most productive plants and then isolate microorganisms from their large nodules. This approach is quite functional, but, in our opinion, it is not without its drawbacks. The fact is that by the time plants can already be selected by phenotype, they find themselves at that stage of the growing season when, according to the data we received, they have already undergone selection of the most suitable variants of nodules. In this case, the plant probably chose nodules with optimal nitrogen-fixing activity for it, that is, not maximum, but sufficient for normal growth. However, for crop production it is necessary to obtain ultra-high yields, which can be facilitated by artificial inoculation of legumes. In this case the plant can be forced to use highly effective strains to form most of the nodules. The higher the efficiency of the bacteria, the higher the yield of legumes. As we assume, under normal conditions, plants during their development prefer to get rid of single overactive nodules that they formed at the very beginning of the growing season, since such nodules are not beneficial to the plant itself, especially if the growing conditions are not entirely favorable. Such conditions include edaphic, climatic, and environmental factors. Therefore, the most productive, in our opinion, will be the search for highly effective strains in nodules formed at the very beginning of the growing season of the host plant. In this case, there is a possibility of detecting microorganisms with the highest nitrogen-fixing activity, which will simply be lost by the middle of the growing season. In addition,

it may be successful to search for effective rhizobia in autumn nodules of perennial plants. In this case, the plant most likely ensures an increase in the number of rhizobia, so that they, having moved into the soil from their structures, become candidates for the next interaction in the new season. At the same time, since trophic connections no longer play a significant role, bacteria with different nitrogen fixation efficiencies may appear in the nodules, and there is a possibility of discovering very promising forms among them.

To summarize, in the three species of legumes we studied, the *Lotus corniculatus* L., *Galega orientalis* Lam. and *Trifolium hybridum* L., at the beginning of the growing season the nodules are formed by the most diverse strains of rhizobia with a high scatter of nitrogen-fixing activity values. During this period, highly effective strains are most often found among isolates. In the middle of the growing season, bacteria have a relatively equal efficiency of nitrogen fixation, while their nitrogen-fixing activity is lower than that of strains isolated at the beginning of the growing season. At the end of the plant life cycle, the composition of rhizobial isolates is quite diverse, but in terms of nitrogen fixation efficiency, they are inferior to strains from the nodules of young plants. Thus, we have shown for the first time that for the most productive search for highly effective strains of nodule bacteria, not only the phenotype is important (we absolutely do not dispute the value of this parameter), but also the stage of plant development. In our opinion, the best chance of finding the desired strains is at the beginning of the development of a legume plant among the first formed nodules.

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