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# EFFECTIVENESS OF NITROGEN-FIXING SYMBIOSIS OF GUAR (*Cyamopsis tetragonoloba*) WITH STRAINS *Bradyrhizobium retamae* RCAM05275 AND *Ensifer aridi* RCAM05276 IN POT EXPERIMENT

**P.S. ULIANICH[1], A.A. BELIMOV[1] ✉, I.G. KUZNETSOVA[1], A.L. SAZANOVA[1], O.S. YUZIKHIN[1], Yu.V. LAKTIONOV[1], D.S. KARLOV[1], M.A. VISHNYAKOVA[2], V.I. SAFRONOVA[1]**

[1]*All-Russian Research Institute for Agricultural Microbiology,* 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail p.ulianich@gmail.com, belimov@rambler.ru (✉ corresponding author), kuznetsova_rina@mail.ru, anna_sazanova@mail.ru, yuzikhin@gmail.com, Laktionov@list.ru, makondo07@gmail.com, v.safronova@rambler.ru;
[2]*Federal Research Center Vavilov All-Russian Institute of Plant Genetic Resources,* 42-44, ul. Bol'shaya Morskaya, St. Petersburg, 190000 Russia, e-mail m.vishnyakova.vir@gmail.com

ORCID:
Ulianich P.S. orcid.org/0000-0002-2768-505X
Belimov A.A. orcid.org/0000-0002-9936-8678
Kuznetsova I.G. orcid.org/0000-0003-0260-7677
Sazanova A.L. orcid.org/0000-0003-0379-6975
Yuzikhin O.S. orcid.org/0000-0002-1818-9230
Laktionov Yu.V. orcid.org/0000-0001-6241-0273
Karlov D.S. orcid.org/0000-0002-9030-8820
Vishnyakova M.A. orcid.org/0000-0003-2808-7745
Safronova V.I. orcid.org/0000-0003-4510-1772

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## Abstract

Legume plant guar (*Cyamopsis tetragonoloba* (L.) Taub.) is a source of guar gum, a complex of polysaccharides that is used in various industries. This crop is widely cultivated mainly in India and Pakistan, but in recent years there has been an increasing interest in the industrial cultivation of guar in the southern regions of Russia. One of the problems of introducing this culture into Russian agriculture is the absence in the soil of bacteria that can form symbiotic nodules on the roots of guar in the soil-climatic conditions of the Russian Federation. One of the problems of the introduction of this crop into the agriculture of the Russian Federation is the absence of bacteria capable of forming symbiotic nodules on guar roots under environmental conditions of Russia. In the present work, the first data on the efficiency of inoculation with nodule bacteria of guar growing in the soils of Russia were obtained. The aim of this work was to evaluate the effectiveness of promising rhizobial strains of guar *Bradyrhizobium retamae* RCAM05275 and *Ensifer aridi* RCAM05276 when growing plants in soils selected in the regions of the Russian Federation and not containing the corresponding nodule bacteria. To inoculate the seeds of guar variety Kubanskiy Yubileiny, inoculums in the form of aqueous suspensions of bacteria were used. Inoculums were obtained according to the standard procedure for the preparation of liquid biopreparations of nodule bacteria. The effectiveness of symbiosis was studied in a pot experiment with growing plants in sod-podzolic soil and chernozem. Inoculation with both strains resulted in active formation of nodules (about 20-40 nodules per plant), while no nodules were found on the roots of control uninoculated plants. The number of nodules per plant was maximal in the variants of inoculation with the strain *B. retamae* RCAM05275. The values of the total mass of nodules per plant were maximum in the variants of inoculation with the strain *E. aridi* RCAM05276 due to the formation of larger nodules. The nodules formed on lateral roots had a rounded irregular shape, pinkish color (evidence of the presence of leghemoglobin in them) and significantly varied in size. Both strains increased the biomass of shoots and the whole plant by about 70% when growing plants on sod-podzolic soil and chernozem, but did not affect the biomass of the roots. The inoculated plants had approximately the same nitrogenase activity regardless of the rhizobia strain and soil type. The specific nitrogenase activity (per nodule biomass) was approximately 2 times higher than in other variants when plants were inoculated with the strain *B. retamae* RCAM05275 in sod-podzolic soil. In all variants of the experiment, an approximately 1.4-fold increase in the total nitrogen content and a 3-4-fold increase in nitrogen accumulation in the shoots of inoculated plants were revealed. Thus, the first data on the efficiency of inoculation with nodule bacteria of the guar cultivated in Russian soils have been obtained. Both studied strains were able to form nitrogen-fixing symbiosis, which led to a significant increase in plant biomass and accumulation of nitrogen in shoots. The results showed the promise of further research on testing strains in field experiments in order to create biopreparations to improve the nitrogen nutrition of this crop.

Keywords: nitrogen fixation, guar, nodulation, symbiosis, *Cyamopsis tetragonoloba*

Guar *Cyamopsis tetragonoloba* (L.) Taub. is an important tropical legumes, the seeds of which contain guar gum used in the coal, oil, gas, food, textile, paper and cosmetic industries. Guar is widely cultivated in India and Pakistan, and also in Afghanistan, Kenya, Australia and the semi-desert regions of the USA [1]. Demand for guar gum is constantly growing, and in 2016, imports of guar gum to Russia exceeded 15 thousand tons [2]. Guar was brought to Russia in the mid-1920s [3], but did not find wide distribution due to insufficient knowledge about the technology of cultivation [4]. In recent years, interest in the commercial growing of guar in the Southern Russia has been growing [5]. A way to address the chellenge is searchinf for strains of nodule bacteria (rhizobia) capable of forming an effective nitrogen-fixing symbiosis with guar in the soil under climatic conditions of the Russian Federation and to procude novel biological preparations based on these microorganisms.

Seed inoculation with biopreparations of nodule bacteria provides intensive biological nitrogen fixation which enhances photosynthesis and increases the yield of legumes [6, 7]. The use of rhizobia for inoculation is especially important when cultivating legumes in new areas, the soils of which do not contain the necessary microsymbionts. For example, when trying to grow soybean *Glycine max* (L.) Merr. in geographic zones of Russia atypical for the species, nodules were practically not formed on the roots. Therefore, it was necessary to develop biological preparations and inoculate seeds with specific rhizobia [6]. Currently, to solve this problem, soybean seed producers provide their supply together with nodule bacteria biopreparations (https://kingsagriseeds.com/soybeans/).

Slowly growing root nodule bacteria of the genus *Bradyrhizobium* (family *Bradyrhizobiaceae*) are the main group of rhizobia entering into symbiosis with guar [8, 9]. In most cases, the strains were not identified to species level but many were close to *B. japonicum* [10, 11]. This species includes a very large and genetically heterogeneous group of soybean microsymbionts, as well as strains that nodulate different types of cowpea (*Vigna*), lupine (*Lupinus*), seradella (*Ornithopus*) and a wide range of leguminous plants of the genistoid complex [12-14].

Inoculation of guar with strains of *Bradyrhizobium* spp. has a positive effect on plant development, significantly increases the number of nodules (up to 79%), plant weight (up to 71%), root weight (up to 262%), seed yield (up to 53%), protein content (up to 33%), fiber (up to 26%), increases the total content of nitrogen and minerals [8, 12, 15-17]. The ability of guar to form symbiosis with fast growing rhizobia *Ensifer aridi* (family *Rhizobiaceae*), which also have a wide range of leguminous hosts from the subfamilies *Mimosoideae* and *Papilionoideae*, has recently been described [18]. The presence of representatives of the genera *Bradyrhizobium* and *Ensifer* among guar nodule bacteria is common to this plant, soybean and cowpea [19-21].

Along with the creation of new varieties and development of special agrotechnologies, including mineral fertilizing, successful introduction of guar in Russia requires the selection of effective microsymbionts to create biological preparations for the crop. In India, the use of mineral nitrogen fertilizers is limited by the high cost and low level of agricultural mechanization. Therefore, the high productivity of guar is achieved largely due to the presence of highly effective nodule bacteria strains in soils. It should be emphasized that in the regions of Russia where guar cultivation has recently begun (Krasnodar Territory, Crimea, Astrakhan and Volgograd regions, and Dagestan) no symbiotic nodules appear, as it is reportef by Rusian breeders.

Previously, we isolated guar nodule bacteria, studied their biodiversity, and as a result characterized slow-growing strains of the genus *Bradyrhizobium* [22] and fast-growing strains of the genus *Ensifer* (unpublished data).

The purpose of this work was to assess the capability of effective symbiosis in two promising strains of rhizobia (the genera *Bradyrhizobium* and *Ensifer*) with guar plants grown in pots with soil samples from different regions of Russia lacking complementary nodule bacteria.

*Materials and methods.* Nodule bacterium strains *Bradyrhizobium retamae* RCAM05275 [23] and *Ensifer aridi* RCAM05276 [24] isolated from guar nodules, were characterized in preliminary experiments, patented by the authors, and deposited (the Departmental Collection of Agricultural Useful Microorganisms, ARRIAM, St. Petersburg). For seed treatment with bacteria, an inoculum prepared in the laboratory as a liquid sample was used. The pure bacterial inoculum was placed in flasks with 250 ml of a semi-synthetic nutrient medium with (g/l) mannitol, 10; yeast extract 1; $K_2HPO_4$ 0.5; $MgSO_4 \cdot 7H_2O$ 0.2; NaCl 0.1. The cultures were incubated at 28 °C on a shaker at 180 rpm for 3 days (*E. aridi* RCAM05276) and for 5 days (*B. retamae* RCAM05275). With standard asepsis precautions, the contents of the flask were transferred into a laboratory fermenter BIORUS 5L (BIORUS, Russia) containing 5 l of the same medium. The fermentation was performed at 28 °C with aeration of 1 l air $\cdot$ 1 l medium$^{-1} \cdot$ min$^{-1}$ and stirring at 200 rpm for 3 days (*E. aridi* RCAM05276) or 4 days (*B. retamae* RCAM05275). Then the bacterial suspensions (inoculum) were placed in a sterile container and stored at room temperature (22-24 °C) for later use.

Seeds of the guar *Cyamopsis tetragonoloba* (L.) Taub. cv. Kuban Yubileiny (Vavilov All-Russian Institute of Plant Genetic Resources St. Petersburg) were scarified and surface sterilized in 98% $H_2SO_4$ for 30 min, washed with sterile tap water and germinated on filter paper in Petri dishes at 25 °C in the dark for 2 days. For the pot test, we used soddy-podzolic soil (Albic Retisol, Abruptic, Ochric, Pskov Province) and chernozem (Haplic Chernozem, Pachic, Voronezh Province) sampled in the summer of 2017. Soddy-podzolic soil was provided by the Pskov Research Institute of Agriculture and the Rodina state farm (57°50′44.2″ N, 28 12′03.7″ E), chernozem was obtained in the Kamennaya Steppe reserve (51°01′41.6″ N, 40 43′39.3″ E). Agrochemical parameters of dry soil were measured according to standard methods [25]. The soil was introduced into 400 g metal pots and fertilized with $K_2HPO_4$ (600 mg/kg). The germinated seeds were exposed to the inoculum for 1 h and planted (2 pots, 4 seedlings for each treatment). Plants were grown for 106 days in a phytoroom with a relative humidity of 60% at a 2-level light and temperature regimes, darkness, 18°C, 8 h for night, 400 μlx $\cdot$ m$^{-2} \cdot$ s$^{-1}$, 23 °C, 16 h for day. DNaT lamps (OOO SSZ Lisma, Russia) and L36W/77 FLUORA lamps (OSRAM Licht AG, Germany) were used for illumination. Soil moisture was maintained at the level of 60-70% of the total moisture capacity by regular watering while weighing the pots. On day 45, the soil was additionally fertilized with $K_2HPO_4$ solution (600 mg/kg).

At the end of the experiment, the plant roots together with the formed nodules were separated from the shoots, washed with tap water, placed in sealed plastic test tubes, and acetylene (5% of the tube volume) was added to measure biological nitrogen fixation by the acetylene method. To do this, after incubating the tubes at 25 °C in the dark for 1 h, the amount of ethylene was determined on a GC-2014 gas chromatograph with a FID detector and a SUS 2.0 m×3.0 mm (L×ID) column (Shimadzu Corporation, Japan). Analytical parameters: detector temperature +250 °C; nitrogen as a carrier gas; the nitrogen flow rate 70 ml/min; injector temperature +72 °C; column temperature +70 °C; analysis time — 5.0 min; ethylene retention time 1.20±0.01 min; acetylene retention time 2.00±0.01 min.

Roots were photographed using a PC1742 camera (Cannon, Japan) and nodules were photographed using a Stemi 508 stereomicroscope (Carl Zeiss, Germany).

The nodules were separated from the roots and cpunted for each plant. Shoots, roots, and nodules were dried at room temperature and weighed. The content of total nitrogen in the shoots was measured (a Kjeltec 8200 automatic analyzer, FOSS Analytical, Denmark) according to the manufacturer's standard method.

Statistical analysis of data was performed using STATISTICA v. 10 (TIBCO Software Inc., USA). Differences between mean values were assessed using one-way analysis of variance and Fisher's LSD test, and the equality of sample variances was checked using Levene's Test.

*Results.* The table shows agrochemical parameters of the soil samples we used in the experiment.

**Agrochemical parameters of soils used in the experiment**

| Parameter | Sod-podzolic | Chernozem |
|---|---|---|
| Mass fraction of organic matter, % | 2.4 | 8.8 |
| Nitrogen total, % | 0.22 | 0.38 |
| Ammonia nitrogen, mg N/kg | 25 | 37 |
| Nitrate nitrogen, mg N/kg | 9.6 | 26.3 |
| Mobile phosphorus, mg $P_2O_5$/kg | 85 | 121 |
| Mobile potassium, mg $K_2O$/kg | 60 | 155 |
| Hydrolytic acidity, mmol/kg | 29 | 18 |
| The sum of absorbed $Ca^{2+}$, $Mg2^{+}$, $Na^{+}$, $K^{+}$, $NH^{4+}$, mmol/kg | 60 | 372 |
| $pH_{H2O}$ | 6.1 | 7.3 |
| $pH_{KCl}$ | 4.9 | 6.2 |

Note. Soddy-podzolic soil and chernozem were selected in 2017, respectively, in Pskov (57°50′44.2″ N, 28°12′03.7″ E) and Voronezh (51°01′41.6″ N, 40°43′39.3″ E) regions. The mass fraction of organic matter was measured by Tyurin's method; total nitrogen by Kjeldahl method; ammonia nitrogen with Nessler's reagent (extraction with 2% KCl solution); nitrate nitrogen as per the disulfophenol method (extraction with 0.05% $K_2SO_4$ solution); mobile phosphorus by formation of phosphomolybdenum blue, mobile potassium by the flame photometric method; extraction from soddy-podzolic soil was carried out with 0.2 mol/l HCl, from chernozem with 10 g/l $(NH_4)_2CO_3$; hydrolytic acidity and the amount of absorbed bases were measured by the Kappen method using extraction with 1 N. $CH3COONa \cdot 3H_2O$ and 0.1 N. HCl, respectively.

The inoculation of guar with both strains led to the active formation of nodules (approx. 20-40 per plant, Fig. 1, A), while no nodules were found on the roots of the control plants. When growing plants on chernozem, the number of nodules formed by *B. retamae* RCAM05275 was 2.8 times higher than in plants grown on soddy-podzolic soil and inoculated with *E. aridi* RCAM05276. The total weigh of nodules per plant was maximum with *E. aridi* RCAM05276 on soddy-podzolic soil and chernozem, but the differences between the test options were not significant due to wide variation in this parameter (see Fig. 1, B).

It has previously been shown that nodulation in guar can be inhibited by high concentrations of available soil nitrogen (26). In the chernozem we used, the content of various forms of nitrogen was 1.5-2 times higher than in the soddy-podzolic soil (see Table). However, this did not lead to the inhibition of nodule formation, which indicates the ability of both partners to form symbiosis at different levels of soil nitrogen.

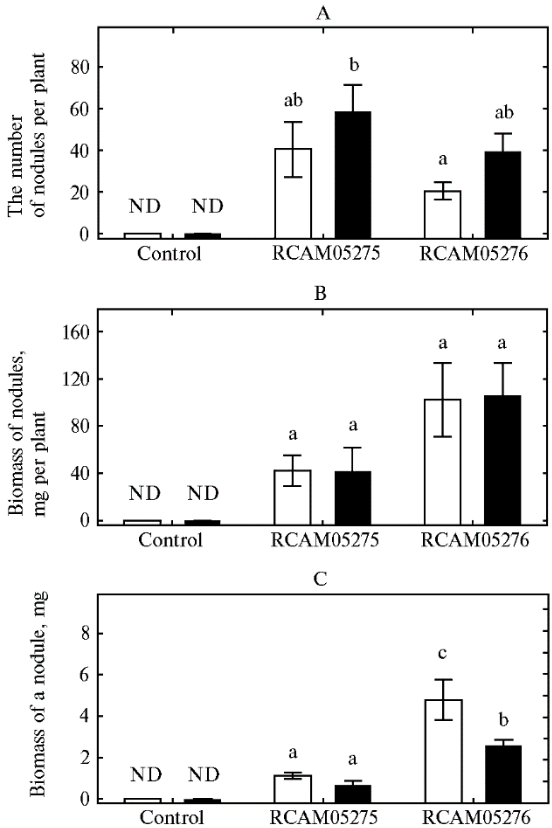


**Fig. 1. The number of nodules** (A) **and the dry biomass of nodules (B) per plant and the biomass of one nodule (C) on the roots of guar *Cyamopsis tetragonoloba* (L.) Taub. cv. Kuban Yubileiny:** control — without inoculation, RCAM05275 — inoculation with the strain *Bradyrhizobium retamae* RCAM05275, RCAM05276 — inoculation with the strain *Ensifer aridi* RCAM05276; white columns — sod-podzolic soil, black columns — chernozem. Vertical bars indicate mean error, ND — not detected. Statistically significant differences are marked with different Latin letters ($n$ = 8, Fisher's LSD test, $p < 0.05$; lab experiment).

As compared to *B. retamae* RCAM05275, the strain *E. aridi* RCAM05276 can form nodules larger in size (see Fig. 1, C). The largest nodules in the strain *E. aridi* RCAM05276 were formed on soddy-podzolic soil. Information on the effect of soil type and composition on the size of symbiotic nodules and the role of the rhizobia strain in this dependence is very limited.

It is known that the plant controls the formation (number and biomass) and functioning (photosynthate influx and transport of nitrogen compounds) of nodules [27]. The number and biomass of formed nodules also vary significantly depending on the microsymbiont strain [6, 28, 29]. The processes of symbiosis formation depend on the physicochemical properties of the soil, such as the content of organic matter, nitrogen available to plants, acidity, and other factors [29, 30]. Aboriginal rhizospheric microorganisms, which modulate the hormonal status and supply of plants with nutrients and interact with introducers, have a great influence on the number of nodules formed [31]. The observed phenomenon of soil influence on nodule biomass can be associated with several of the listed factors and its explanation requires a more detailed study.

Both strains increased the biomass of shoots by approx. 70% when growing guar on soddy-podzolic soil and chernozem (Fig. 2, A), but did not affect the biomass of roots (see Fig. 2, B). As a result, the biomass of the entire inoculated plant in all variants of the experiment was also greater than in the control, by about 60-80% (see Fig. 2, C).

This is consistent with the literature data on the high responsiveness of guar to inoculation with nodule bacteria, which manifested itself in an increase in the biomass of the aboveground parts of plants and seed yield [8, 11, 13].

Figure 3, as an example, shows the appearance of the above-ground part, the root system and nodules of plants grown on soddy-podzolic soil on the day the experiment was completed.

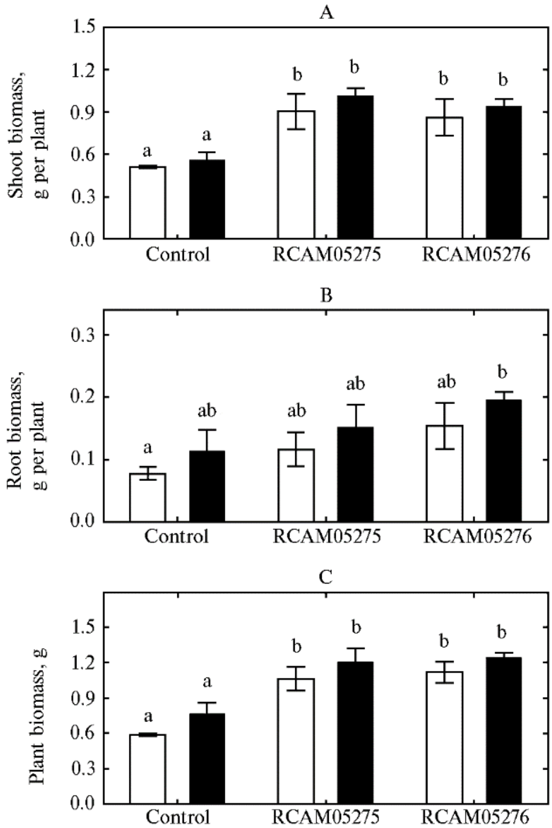


**Fig. 2. Dry biomass of shoots** (A), **roots** (B) **and total plant biomass** (C) **of guar *Cyamopsis tetragonoloba* (L.) Taub. cv. Kuban Yubileiny:** control — without inoculation, RCAM05275 — inoculation with the strain *Bradyrhizobium retamae* RCAM05275, RCAM05276 — inoculation with the strain *Ensifer aridi* RCAM05276; white columns — sod-podzolic soil, black columns — chernozem. Vertical bars indicate mean error, ND — not detected. Statistically significant differences are marked with different Latin letters ($n$ = 8, Fisher's LSD test, $p < 0.05$; lab experiment).

The control plants were significantly inferior to the inoculated plants in terms of leaf height and area, and showed signs of chlorosis, probably due to nitrogen deficiency (Fig. 3, A). The roots were well developed, branched, but had no nodules (see Fig. 3, B). Both strains formed nodules on lateral roots, with single nodules and groups of closely spaced nodules occurring (see Fig. 3, C, E). The nodules of both strains had an irregular round shape and significantly varied in size (see Fig. 3, D, F). The pinkish color of the nodules indicates the presence of leghemoglobin which is necessary for atmospheric nitrogen fixation.


**Fig. 3. Appearance of shoots** (A), **roots** (B, C, E) **and nodules** (D, F) **of guar *Cyamopsis tetragonoloba* (L.) Taub. cv. Kuban Yubileiny plants grown on soddy-podzolic soil:** B — control without inoculation,

C, D — inoculation with *Bradyrhizobium retamae* strain RCAM05275, E, F — inoculation with *Ensifer aridi* RCAM05276 strain (lab experiment).

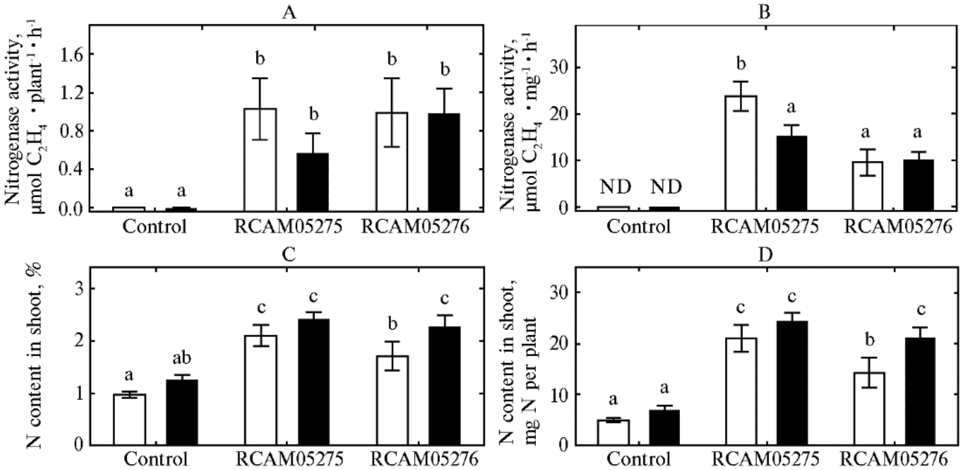


**Fig. 4. Nitrogenase (acetylene reductase) activity** (A)**, specific nitrogenase activity per nodule biomass unit** (B)**, content** (C) **and accumulation** (D) **of total nitrogen in guar *Cyamopsis tetragonoloba* (L.) Taub. cv. Kuban Yubileiny shoots:** control — without inoculation, RCAM05275 — inoculation with the strain *Bradyrhizobium retamae* RCAM05275, RCAM05276 — inoculation with the strain *Ensifer aridi* RCAM05276; white columns — sod-podzolic soil, black columns — chernozem. Vertical bars indicate mean error, ND — not detected. Statistically significant differences are marked with different Latin letters ($n$ = 8, Fisher's LSD test, $p < 0.05$; lab experiment).

The inoculated plants had approximately the same nitrogenase activity regardless of the rhizobia strain and soil type (Fig. 4, A). But the specific nitrogenase activity, expressed per unit of nodule biomass, was 1.5-2.4 times higher in soddy-podzolic soil when plants were inoculated with B. etamae RCAM05275 than in other variants (see Fig. 4, B). It is likely that the relatively low biomass of nodules (see Fig. 1, B) was compensated by an increase in the efficiency of the nitrogen fixing system with the participation of the B. retamae RCAM05275 strain. Strain comparisons and measurement of nitrogen fixation activity were not performed in these studies, but nodule mass was previously shown to correlate with nitrogen accumulation and yield when comparing 50 guar genotypes [32]. Little is known about the level of nitrogenase activity of guar nodules, but the values obtained by us were comparable with the data on the measurement of nitrogenase activity in soybean inoculated with various strains of the genera Bradyrhizobium and Ensifer [33, 34].

The effective functioning of the symbiosis was also indicated by an increase in the total nitrogen content by about 1.4 times (see Fig. 4, C) and nitrogen accumulation by approx. 3-4 times (see Fig. 4, D) in the shoots of inoculated guar plants. These effects manifested themselves in all variants of the experiment with a minimum value during the inoculation of plants grown on soddy-podzolic soil with the E. aridi RCAM05276 strain. A significant increase in the content and accumulation of nitrogen in guar plants as a result of inoculation with nodule bacteria has been repeatedly described [8, 11, 12, 35]. It was also shown that strains of the genus Bradyrhizobium fixed nitrogen more actively in symbiosis with soybean than strains of the genus *Ensifer* [34]. In our experiments, B. retamae RCAM05275 was not inferior to the *E. aridi* RCAM05276 strain in terms of the measured parameters of symbiosis, and on soddy-podzolic soil it increased the content and accumulation of nitrogen in plants by 26% compared with the increase in these parameters under the influence of the *E. aridi* RCAM05276 strain (see Fig. 4, C, D). On average, for all variants of the experiment, the biomass of an individual nodule negatively correlated with the nitrogen content in the shoots ($r$ = 0.98; p = 0.019; $n$ = 4), which indicates a higher efficiency of small nodules formed by the strain B. retamae RCAM05275.

Thus, both strains, *B. retamae* RCAM05275 and *E. aridi* RCAM05276, can form effective symbiosis with guar when growing plants in soddy-podzolic soil and chernozem, selected in different regions of the Russian Federation and not containing complementary nodule bacteria. The strains were similar in terms of nodule formation and symbiosis efficiency. However, the characteristic features of strains in interaction with guar plants were also revealed, which was expressed in differences in the number of nodules, specific nitrogenase activity, and intensity of nitrogen supply to plants. Our results showed the promise of further testing of the studied strains in field experiments in order to create biological preparations to improve the nitrogen nutrition of guar plants.

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