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Nodulation in Onobrychis Perennial Legume Plants

Zair S. Shakirov[1*], Sardor A. Khakimov[1], Khabibullo F. Shomurodov[2], Bakhtiyar R. Umarov[1]

[1]Institute of Microbiology of Uzbekistan Academy of Sciences, Tashkent, Republic of Uzbekistan; [2]Scientific Center of Plant Production "Botanika", Uzbekistan Academy of Sciences, Tashkent, Uzbekistan.
Email: *zair@dostlink.net

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ABSTRACT

A total of 110 *strains of nodule bacteria was isolated from plants Onobrychis transcaucasica and Onobrychis chorassanica. Nodulation study of bacteria in both Onobrychis plant species in microvegetation experiment gave a very low nodulation on plant roots. The intensive nodulation of Onobrychis plants was recorded in vegetation experiment and for Onobrychis transcaucasica the efficiently-nodulating strains were found OT*102, *OT*103, *OT*117, *OT*121, *OT*130, *OT*136, *OT*139, *OT*140, *while for Onobrychis chorassanica plants – OC*106, *OC*107, *OC*109, *OC*112, *OT*103, *OT*117 *and OT*123 *strains. Nucleotide sequencing of the 16S rRNA gene and BLAST analysis showed that nodule bacteria of Onobrychis plants were related to Rhizobium, Burkholderia, Enterobacter and Pantoea genera. It has been shown a possibility of growing up of Onobrychis plants at minimal additional moisture of sabulous soils in the Kyzyl Kum Desert, creating artificial pastures and thereby immobilizing the desert blown sands.*

Keywords: *Onobrychis Transcaucasica*, *Onobrychis Chorassanica*, *Nodulation*, *Nitrogen Fixation*, 16*S rRNA*, *Rhizobium*, *Burkholderia*, *Enterobacter*, *Pantoea*

1. Introduction

Although more than on 32 millions Ha world wide alfalfa is grown, it is conducted a search for new special purpose forage legumes supported by smaller plantings of species of *Coronilla*, *Onobrychis*, and *Lotus* [1].

Actually, there was tried an *Onobrychis* inoculation experiment, but due to too low germination rates of seeds and poorer nodulation, no reliable data were obtained [2]. Rhizobia from Canadian soils were selected for cold adaptation with the aim of improving productivity of legumes that are subjected to cool temperatures during the growing season [3]. One approach was to use rhizobia associated with legume species indigenous to arctic and subarctic regions: *Mesorhizobium* sp. isolated from *Astragalus*, *Oxytropis* spp. and *Rhizobium leguminosarum* from *Lathryrus* spp. The majority of these rhizobia are considered as psychrotolerant because they can grow at 0℃. The advantages of cold adaptation of arctic *Mesorhizobium* to improve legume symbiosis were demonstrated with the temperate forage legume sainfoin (*Onobrychis viciifolia*). In laboratory and field studies, arctic rhizobia were more efficient than temperate (commercial) rhizobia in improving growth of sainfoin and were more competitive in forming nodules. Biochemical studies on cold adaptation showed higher synthesis of cold shock proteins in cold-adapted than in non-adapted arctic rhizobia. Since arctic *Mesorhizobium* cannot nodulate agronomically important legumes, the nodulation genes and the bacterial signals (Nod factors) were characterized as a first step to modifying the host specificity of nodulation [3].

The genetic diversity of 44 rhizobial isolates from *Astragalus*, *Oxytropis*, and *Onobrychis* spp. originating from different geographic locations was evaluated by mapped restriction site polymorphism (MRSP) analysis of 16S rRNA genes and by PCR DNA fingerprinting with repetitive sequences (REP-PCR) [4]. From the REP-PCR data, authors identified only three examples in which rhizobia from a single plant species appeared to be closely related (the two strains from *Astragalus sinicus*, two strains from *Onobrychis viciifolia*, and three strains from *Astragalus cicer*). These results agree with previous classifications of other publications by serology, by numerical taxonomy, and by cross-infection experiments in which rhizobia from *Astragalus*, *Oxytropis*, and *Onobrychis* spp. were grouped independently of their plant

origin [4].

Five isolates from sainfoin (*Onobrychis viciifolia*, tribe *Hedysareae*, related to the tribe of *Galegeae*) were included because this legume species was effectively nodulated by rhizobia isolated from *Astragalus* and *Oxytropis* [5]. The nitrogenase activities of five arctic rhizobia isolates were higher at low temperatures than those of temperate rhizobia when symbiotic with the legume sainfoin (*Onobrychis viciifolia* Scop.) [6]. It was also found that a strain isolated from a cold environment caused nodulation and formed bacteroids under low temperatures while a strain isolated from a warmer environment did not [7].

Since *Phaseolus vulgaris* is a promiscuous host nodulated by at least six species of rhizobia, introduced plants could also have established symbiosis with rhizobia from *Leucaena, Onobrychis, Dalea*, etc. [8], with other words, nodule bacteria isolated from *Onobrychis* plants could display cross-inoculation host specificity towards to the strange host-plant.

The tasks of the present research included a study of symbiotic properties of 110 nodule bacteria isolated from nodules of *Onobrychis transcaucasica* and *Onobrychis chorasanica,* as well as their generic and specific (species) belonging, and also the sand immobilization with help of *Onobrychis* symbiosis aiming to create artificial semi-desert pastures and increase their productivity.

2. Materials and Methods

2.1. Isolation and Purification of Nodule Bacteria from *Onobrychis* Plants

Nodules with pink and fallow tissue were taken from the root system of both *Onobrychis* species. The nodules were surface-sterilized with 30% H_2O_2 for 30 s and washed several times with sterile distilled water [9]. Sterile nodules were crushed gently up to homogenous state and the nodule contents were streaked on medium of the following composition (g/L): glucose—5, sucrose —5, K_2HPO_4—0.5, KH_2PO_4—0.5, $MgSO_4 \cdot 7H_2O$—0.5, $CaSO_4$—0.2, pea—50, agar—20, water distilled - up to 1 L, pH 6.8-7.0 (pea was boiled during 1 hour and the medium was prepared on the basis of pea's broth) [10]. The bacteriological-pure nodule bacteria were isolated from single grown colonies.

2.2. Microvegetation and Vegetation Experiments

For microvegetation experiments *Onobrychis* seeds were treated by concentrated sulphuric acid during 4 minutes, then after their numerous washing by sterile water the treated seeds were put on sterile wet discs from filter paper into Petri dishes and were incubated in thermostat at 30℃ temperature for 1-2 days before their germination. The germinated seeds further were introduced into tubes with volume 60 ml with sterile mixture sand: vermiculite (3:1), height of which was 8 cm, containing nutritive medium for plants, into each tube there was added 8 ml of this medium: $MgSO_4 \cdot 4H_2O$—5 mM, K_2SO_4—10 mM, $CaCl_2 \cdot 2H_2O$—1 mM, phosphate buffer (NaH_2PO_4 + Na_2HPO_4, pH 6.5)—15 mM, Fe-Sequestrene 138 (Fe-EDDHA)—5mM, microelements—0.05 ml/L of medium. Microelements (g/L): H_3BO_3—17.16, $MnSO_4$—7.2, $ZnSO_4$—1.32, $CuSO_4$—1.65, Na_2MoO_4—0.12 [11].

In vegetation experiment the plants were grown within bags of 2 L volume that were filled by sand impregnated with nutritive medium. After appearance of seedlings of germinated seeds, they were inoculated with bacterial suspensions of 3-daily nodule bacteria cultures that were prepared in the nutritive medium solution in titre 10^9 cells/ml (on 2 ml of microelements solution per each tube together with 10 ml of bacterial suspension in the nutriative medium per each bag). In microvegetation experiment the plant seedlings were inoculated by all isolates that were isolated from nodules of both *Onobrychis* plant species. In vegetation experiment the plant seedlings were inoculated by *Onobrychis transcaucasica* isolates (OT102, OT103, OT111, OT114, OT115, OT117, OT118, OT121, OT122, OT123, OT124, OT130, OT136, OT139, OT140, OT148, OT151) and *Onobrychis chorassanica* isolates (OC104, OC106, OC107, OC109, OC111, OC112, OC113, OC138). The inoculated *Onobrychis* seedlings were cultivated in sterile conditions during 45 days. Each variant of inoculation was done in 3 repeats on 2 plants per each repeat (in vegetation experiment - 5 plants/repeat).

2.3. Determination of Nitrogen-Fixing Activity

Nitrogen-fixing activity was estimated by the acetylene-reductase activity (ARA) assay described by Hardy [12]. The plant samples (with root nodules) were washed with sterile water and transferred into 60 ml capacity agronomic tubes fitted with airtight rubber stoppers. Acetylene (10 volume %) was injected and the tubes were incubated at 30°C for 24 hours. The data was the mean of three replicates. The samples without acetylene were used as control. The quantitative estimation of ethylene gas produced in the samples was measured on a gas chromatograph (LHM-80). The acetylene-reductase activity of the plants was expressed as nmoles C_2H_4 / tube/hour.

2.4. PCR Amplification of the 16S rRNA Gene

The 16S rRNA gene from nodule bacteria of *Onobrychis transcaucasica* and *Onobrychis chorassanica* was amplified using universal primers 1070f (59-ACGGGCGGTG

TGTAC-39) and 1392r (59-CGCCCGCCGCGCCCCGC GCCCGGCCCGCCGCCCCCGCCCCACGGGCGGTG TGTAC-39) [13]. Each PCR mixture contained the following: 10 pmol each primer, 200 μM dNTPs, 1U Tag DNA polymerase, 100-200 ng genomic DNA and Taq polymerase buffer in a final reaction volume of 50 μl. The DNA thermal cycler used for PCR amplification was programmed as follows: an initial extensive denaturation step at 94℃ for 5 min; 30 cycles of 94℃ for 1 min, 53℃ for 1 min and 72℃ for 1.5 min; and a final extension step at 72℃ for 10 min.

2.5. Phylogenetic Analysis

The complete 300-354-bp 16S rRNA gene sequences were compared with the sequences available in the GenBank database using the standard Basic Local Alignment Search Tool, BLASTn [14], at the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). From the aligned sequences, neighbor-joining dendrograms [15] were constructed with the software MEGA version 4.0.2 [16]. The robustness of the inferred trees was evaluated by 1000 bootstrap resamplings.

2.6. Field Experiments on Sand Immobilization

The plot for field experiments on sand immobilization was chosen in area that was represented by clay (sabulous) sand and all area surrounded experimental plot served as control plot without sowing. A square plot with the area of 400 m^2 was divided into four square subplots of 100 m^2, which in turn were divided into allotments of 10 m length and 0.7 m width. Seeds (3 seeds/hole) were sown at 0.25 m intervals along the length the edge of each allotment. Seeds of *Onobrychis transcaucasica* and *Onobrychis chorassanica* were inoculated with 10^9 cells/ml suspensions of 3-daily grown cultures of bacterial strains as described before [10]. Control plots were sown with non-inoculated seeds.

3. Results

3.1. Isolation of Nodule Bacteria

Onobrychis is related to *Fabaceae* family, *Hedysarea* tribe, *Onobrychis* genus. *Onobrychis chorassanica* is a drought- (xerophyte) and frost-resistant leguminous plant with multiply (up to 20) straight sprouts with height 0.9-1.2 m [17]. *Onobrychis chorassanica* is a steppe plant, which grows in sandy, rubbly, dried zones, on rocky slopes of mountains and foothills and speckled ores of Tien Shan, Pamirs-Alai, Kopetdag, Iranian plateau and in the Northern Afganistan (**Figure 1(a)**). *Onobrychis transcaucasica* is widespread among mountain meadow and steppe areas of Azerbaijan, Georgia and Armenia [18], it

(a)

(b)

Figure 1. The appearance of *Onobrychis chorassanica* (a) and *Onobrychis transcaucasica* (b) plants.

is mezoxerophyte, which was introduced earlier into Uzbekistan (**Figure 1(b)**). For isolation of natural nodule bacteria the nodules were gathered from the roots of *Onobrychis transcaucasica* and Onobrychis *chorassanica* plants, growing up in their natural habitats. There have been isolated 65 nodule bacteria from *Onobrychis transcaucasica* plant nodules and 45 nodule bacteria from *Onobrychis chorassanica* nodules and it has been created a collection of their nodule bacterial isolates. Microscopic investigations of some bacterial cells of

selected strains showed that studied cells were represented with typical motile rods, their width varied from 0.5 to 0.8 micron and length – from 1.0 to 1.5 micron. It was marked a polymorphism of cells in dependence on the cell's age – in logarithmic phase of growth the cells were represented with motile cells, whereas in stationary phase of growth the cells lost their motility. The speed of growth of nodule bacteria isolated strains depends on conditions of cultivation and it is one of the most important taxonomic signs. During this well-formed colony of fast-growing bacteria can be obtained by 3-4 days of growth, but for slowly-growing—by 7-10 days. In media containing agar-agar the isolates of nodule bacteria formed colorless, transparent, slimy colonies. Such type of isolates colonies was relevant to S-forms of bacteria.

3.2. Nodulation Test

The next stage of investigation there was a research of plant nodulation aiming to select high-efficient bacteria in microvegetation experiments. As it was shown in **Figure 2**, the inoculated plants in sterile conditions during microvegetation experiment grew and developed well. Examination of nodulation for *Onobrychis transcaucasica* and *Onobrychis chorassanica* plants on the 45th day of plant growing up showed that nodulation under inoculation with *Onobrychis transcaucasica* isolates (65 isolates) occurred only in 4% from total number of inoculated plants and 13% in *Onobrychis chorassanica* (45 isolates). *Onobrychis transcaucasica* OT102, OT103, OT124, OT148 nodule bacteria formed from 1 to 3 nodules per plant with its original host plant (*Onobrychis transcaucasica,* direct inoculation). At the same time up to 4 nodules were formed on *Onobrychis chorassanica* plant roots during inoculation with both their OC106, OC107, OC109 bacteria (direct inoculation) and *Onobrychis transcaucasica* OT104 bacteria (cross inoculation) (**Figure 2**). From obtained results it may conclude that so low nodulation in both *Onobrychis* species undoubtedly depended on growing conditions, although in sterile micro-vegetation experiments the plants grew well,

Figure 2. Nodulation of Onobrychis transcaucasica (a) and Onobrychis chorassanica (b) under inoculation with nodule bacteria in conditions of sterile microvegetation experiment (45- daily plants): 1 –O T102; 2 – OT148; 3 – OT124; 4 – OT103; 5 –OC104; 6 –OC106; 7– OC109; 8– OC107.

but it was not enough for nodule formation on plant roots. According to literature data in nature the nodulation in *Onobrychis chorassanica* starts by 20-25th day after appearance of sprouts, one week later than in wild species of alfalfa [17]. For getting the high nodulation frequency of *Onobrychis* plants it was necessary to create more optimal growing conditions than it was in microvegetation experiment. In this connection for finding of the most optimal conditions for nodulation the plants of *Onobrychis transcaucasica* (grown in microvegetation experiment during 45 days) inoculated with OT139 nodule bacteria and *Onobrychis chorassanica* plants (grown in microvegetation experiment during 45 days) inoculated by OC140 nodule bacteria were transferred into 2 L bags with sterile sand as potting material. The further growing up of 45-daily plants in vegetation experiment for one month showed that on roots of *Onobrychis* plants formed numerous nodules with size 0.3-0.8 cm and during this the plants developed much better than in microvegetation experiment. In this connection the further experiments on study of nodulation of *Onobrychis* plants were carried out in sterile vegetation experiment. As results showed, the nodulation in both plant species was observed with different nodule number (**Table 1**, **2**; **Figure 3**). The intense nodulation was detected more in *Onobrychis transcaucasica* than in *Onobrychis chorassanica* plants. Among all of tested strains for *Onobrychis transcaucasica* plants OT102, OT103, OT117, OT121, OT130, OT136, OT139 and OT140 strains were efficient (**Table 1**) and for *Onobrychis chorassanica* plants – OC106, OC107, OC109, OC112, OT103, OT117, OT123 strains (**Table 2**). It should be noted that the biggest efficiency for *Onobrychis chorassanica* plants was found under cross inoculation with OT123 strain. The acethylene-reductase activity for *Onobrychis transcaucasica* plants varied within range from 28 to 63 nmoles C_2H_4/hour/tube, while for *Onobrychis chorassanica* – 29-51 nmoles C_2H_4/hour/tube. The correlation between nitrogen fixation and efficiency was observed in symbiosis of *Onobrychis transcaucasica* plants with OT117 and OT139 nodule bacteria, but in *Onobrychis chorassanica* – with OC109 and OT123 The nodule bacteria isolated from nodules of Onobrychis plants displayed cross nodulation specificity (the nodulation specificity towards to non-maternal host plant under cross inoculation of nonmaternal plant) towards to both plant species.

Figure 3. Nodulation of 1-monthly Onobrychis transcaucasica (a) and Onobrychis chorassanica (b) plants under inoculation with nodule bacteria in conditions of sterile vegetation experiments: 1 – OT117; 2 – OT121; 3 – OT123; 4 – OC104; 5 – OC113; 6 – OC107.

Table 1. Nodulation of *Onobrychis transcaucasica* plants under inoculation with nodule bacteria strains isolated from *Onobrychis* plants.

Inoculation variant	Average dry biomass of 1 plant, mg	Average nodule number per 1 plant	ARA, nmoles C_2H_4 /tube/hour	Efficiency symbiosis, %
Control	81.5 ± 2.14	-	-	100
OT102(1)	104.0 ± 3.98	2.0 ± 1.0	46.0 ± 11.0	127.6
OT103	103.0 ± 1.99	2.3 ± 1.5	48.0 ± 4.58	126.3
OT111	98.8 ± 2.27	1.6 ± 1.15	51.0 ± 10,58	121.2
OT114	89.8 ± 5.57	2.6 ± 0.52	49.0 ± 8.18	110.1
OT115	74.8 ± 2.41	2.3 ± 0.57	35.0 ± 4.58	91.7
OT117	111.0 ± 3.97	2.6 ± 1.96	59.0 ± 3.0	136.1
OT118	88.0 ± 1.52	1.3 ± 0.57	48.0 ±7.0	107.9
OT121	101.0 ± 8.08	10.0 ± 2.64	63.0 ± 8.88	123.9
OT122	87.0 ± 6.38	4.6± 1.44	58.0 ± 11.53	106.7
OT123	93.6 ± 6.86	2.3 ± 0.57	51.0 ±.56	114.8
OT124	97.5 ± 6.26	2.6 ± 0.69	53.0 ± 6.0	119.6
OT130	107.0 ± 4.17	2.3 ± 0.51	33.0 ± 14.17	131.2
OT136	102.2 ± 5.38	3.0 ± 1.0	49.0 ± 4.58	125.3
OT139	103.8 ± 5.62	2.3 ± 1.12	57.0 ± 3.0	127.3
OT140	99.6 ± 6.69	3.3 ± 0.51	62.0 ± 9.16	122.2
OT148	84.4 ± 4.22	2.0 ± 1.0	45.0 ± 10.8	103.5
OT151	80.0 ± 3.36	1.6 ± 0.57	28.0 ± 2.48	98.1
OC107(2)	92.6 ± 3.00	2.3 ± 0.57	53.0 ± 5.56	113.6
OC109	79.5 ± 6.47	1.6 ± 1.15	43.0 ± 6.24	97.5
Control	81.5 ± 2.14	-	-	100
OT102(1)	104.0 ± 3.98	2.0 ± 1.0	46.0 ± 11.0	127.6
OT103	103.0 ± 1.99	2.3 ± 1.5	48.0 ± 4.58	126.3
OT111	98.8 ± 2.27	1.6 ± 1.15	51.0 ± 10,58	121.2
OT114	89.8 ± 5.57	2.6 ± 0.52	49.0 ± 8.18	110.1
OT115	74.8 ± 2.41	2.3 ± 0.57	35.0 ± 4.58	91.7
OT117	111.0 ± 3.97	2.6 ± 1.96	59.0 ± 3.0	136.1
OT118	88.0 ± 1.52	1.3 ± 0.57	48.0 ±7.0	107.9
OT121	101.0 ± 8.08	10.0 ± 2.64	63.0 ± 8.88	123.9
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OT124	97.5 ± 6.26	2.6 ± 0.69	53.0 ± 6.0	119.6
OT130	107.0 ± 4.17	2.3 ± 0.51	33.0 ± 14.17	131.2
OT136	102.2 ± 5.38	3.0 ± 1.0	49.0 ± 4.58	125.3
OT139	103.8 ± 5.62	2.3 ± 1.12	57.0 ± 3.0	127.3
OT140	99.6 ± 6.69	3.3 ± 0.51	62.0 ± 9.16	122.2
OT148	84.4 ± 4.22	2.0 ± 1.0	45.0 ± 10.8	103.5
OT151	80.0 ± 3.36	1.6 ± 0.57	28.0 ± 2.48	98.1
OC107(2)	92.6 ± 3.00	2.3 ± 0.57	53.0 ± 5.56	113.6
OC109	79.5 ± 6.47	1.6 ± 1.15	43.0 ± 6.24	97.5

Note: Values are the ±SE, n = 3; ARA – acetylene-reductase activity. OT(1) – nodule bacteria isolated from *Onobrychis transcaucasica* nodules; OC(2) – nodule bacteria isolated from *Onobrychis chorassanica* nodules.

Thus, proceeding from these results one can suppose that more optimal growing conditions which are close to natural conditions of their habitats are necessary for nodulation of Onobrychis transcaucasica and Onobrychis chorasanica plants, because these plants are wild plants.

3.3. Phylogenetic Analysis of the 16S rRNA Gene of Nodule Bacteria Strains

Further, the taxonomy of bacterial isolates of nodule bacteria isolated from nodules of *Onobrychis transcaucasica* and *Onobrychis chorassanica* plants was studied with help of 16S rRNA gene method. The determination of nucleotide sequence of 16S rRNA gene of nodule bacteria of *Onobrychis* plants enabled to realize an identification of specific belonging of the bacteria up to genus, but for some bacteria – up to specie. Results of comparative BLAST analysis of nucleotide sequence of conservative region of 16S rRNA gene of OT102, OT123, OT136, OT140 bacteria from *Onobrychis transcaucasica* were identical with genes of *Rhizobium* sp. EGY2 (AY693662.1) by 99%. It is interesting to note that nucleotide sequences of OT102, OT103, OT111, OT115, OT117, OT123, OT136, OT139, OT140 bacteria from *Onobrychis transcaucasica* have also 96-98% identity with genes of *Sinorhizobium meliloti* CCNWYC140 (EU849576.1), *Sinorhizobium meliloti* (AB535707.1),

Table 2. Nodulation of Onobrychis chorassanica plants under inoculation with nodule bacteria strains isolated from Onobrychis plants.

Inoculation variant	Average dry biomass of 1 plant, mg	Average nodule number per 1 plant	ARA, nmoles C_2H_4/tube/hour	Efficiency symbiosis, %
Control	75.3 ± 3.30	-	-	100
OC104	101.4 ± 8.48	2.6 ± 0.57	42.0 ± 4.58	134
OC106	94.8 ±5.18	1.6 ± 0.57	47.0 ± 6.08	125
OC107	95.6 ± 2.85	1.6 ± 1.15	43.0 ± 7.0	126.9
OC109	97.1 ± 7.01	2.3 ± 1.15	45.0 ± 3.0	128.9
OC111	80.0 ±9.64	1.3 ± 0.51	39.0 ± 3.46	106.2
OC112	90.8 ± 3.63	2.6 ± 1.09	41.0 ± 6.0	120.5
OC113	81.4 ± 6.50	1.3 ± 0.57	37.0 ± 6.24	108.1
OC138	78.6 ± 2.89	1.7 ± 0.64	40.0 ±3.6	104.3
OT102	77.2 ± 4.38	2.3 ± 1.12	43.0 ± 8.88	102.6
OT103	96.8 ± 2.11	3.3 ± 1.15	48.0 ± 3.6	128.5
OT111	92.4 ± 5.93	1.6 ± 0.57	41.0 ± 7.0	122.7
OT115	72.0 ± 6.38	1.3 ± 0.6	29.0 ± 9.84	95.6
OT117	91.8 ± 4.83	1.6 ± 0.69	38.0 ± 2.64	121.9
OT121	75.6 ± 6.61	1.3 ± 1.15	39.0 ± 6.92	100.3
OT123	99.0 ± 5.76	2.3 ± 1.15	46.0 ± 2.64	131.4
OT136	97.5 ± 5.80	2.6 ± 1.52	31.0 ± 6.0	129.4

Sinorhizobium fredii CCBAU 10078 (GU552900.1), *Mesorhizobium mediterraneum* Zw-2-1 (GU201845.1), *Mesorhizobium obiense* Zw-1 (GU201844.1), *Bradyrhizobium japonicum* PRY65 (AF239848.2) bacteria. The conservative region of 16S rRNA gene of OT114, OT124, OT148 bacteria coincides by 98-99% with genes of *Pantoea agglomerans* GS2 (GQ374474.1), *Enterobacter cloacae* IHB B 1374 (GU186117.1) and *Pantoea agglomerans* MKPTK-4 bacteria (GQ499274.1) accordingly.

Analogous results were obtained for nodule bacteria from *Onobrychis chorassanica.* The analysis of 16S rRNA gene of *Onobrychis chorassanica* nodule bacteria showed that nucleotide sequence of OC104, OC107, OC109 and OC111 bacteria by 98- 99% coincides with genes of *Rhizobium* sp. EGY2 (AY693662.1). Moreover, 16S rRNA genes of OC104, OC107, OC109 bacteria by 97-99% were homologous with genes of several bacteria species such as *Sinorhizbium meliloti* CCNWY C140 (EU849576.1), *Sinorhizobium meliloti* YcS2 (AB535707.1), *Sinorhizobium fredii* CCBAU 10078 (GU 552900.1), *Mesorhizobium tianshanense* (FM203306.1), *Mesorhizobium amorphae* CCNWYC131 (EU849577.1) and *Bradyrhizobium japonicum* PRY62 bacteria (AF 239847.2). OC112 bacterium is identical by 99% with nucleotide sequences of 16S rRNA gene of *Burkholderia caryophylli* WAB1944 (AM184283.1), the genes of OC106 coincides by 97% with genes of *Pantoea agglomerans* HXJ (HM016799.1), genes of OC138 by 98% coincides with genes of *Enterobacter* sp. RF-100 (GQ 205104.1) and genes of OC113 bacteria by 97% are identical with genes of *Enterobacter* sp. B-13M3 (AJ 874743.1).

During analysis of phylogenetic tree of created on the basis of nucleotide sequence of 16S rRNA gene of different bacteria it was established that studied OT102, OT103, OT111, OT115, OT117, OT123, OT136, OT139, OT140 bacteria from *Onobrychis transcaucasica* were related to *Alphaproteobacteria* class (**Figure 4(a)**), but OT114, OT124, OT148 bacteria were related to *Gammaproteobacteria* class (**Figure 4(b)**). On phylogenetic tree of *Onobrychis chorassanica* nodule bacteria unlike to *Onobrychis transcaucasica* bacteria formed three clusters. Bacteria, incoming into the 1st cluster, were *Alphaproteobacteria* (**Figure 5(a)**), the 2nd cluster—*Betaproteobacteria* (**Figure 5(b)**), and bacteria of the 3rd cluster were related to *Gammaproteobacteria* class, in particular to *Enterobacter* and *Panoea* genera (**Figure 5(c)**).

3.4. Immobilization of Sand with Onobrychis Plants

The seeds of both *Onobrychis transcaucasica* and *Onobrychis chorassanica* plants were sowed into sabulous sand of experimental desert trial plot; there was an average 17 % of sand humidity at the plot under 3 additional irrigation treatments within 5-6 months. The sprouts emerged already by 2nd-3rd day after seed sowing and they developed up to stage with real (non-embryonal) leaves already by 6-8th day. During the further 5-6 months the plants grew and gave a good yield of green biomass (2 hay cuttings were for the mentioned period) and within this time they formed compact "green belt" of

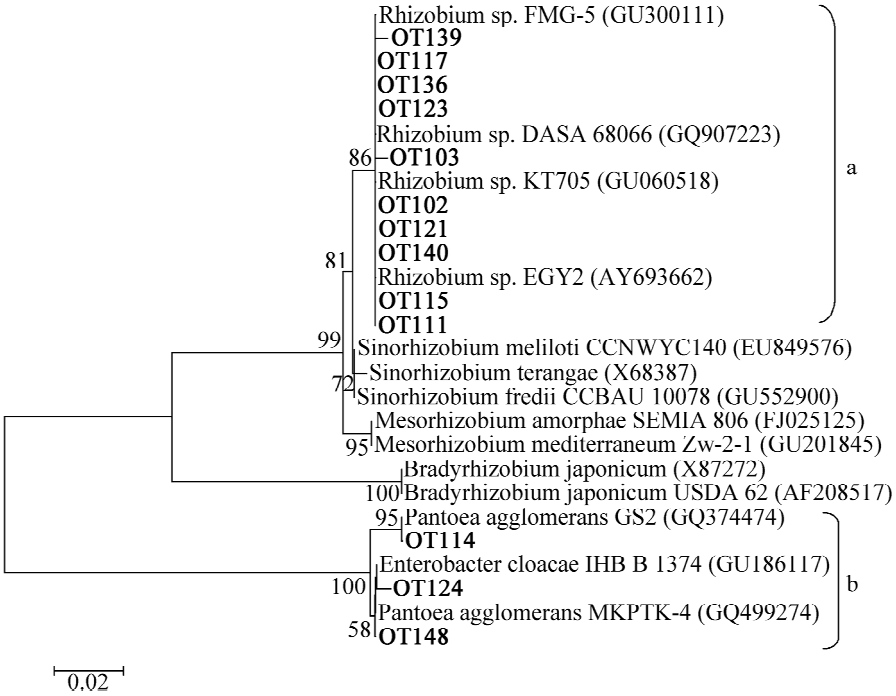

Figure 4. Phylogenetic tree based on the 16S rRNA gene strains of nodule bacteria Onobrychis transcaucasica: (a) Alphaproteobacteria; (b) Gammproteobacteria. The branching pattern was produced by the neighbour-joining method. The GenBank accession numbers for the sequences used are indicated in parentheses. Symbionts of Onobrychis transcaucasica are shown in bold type.

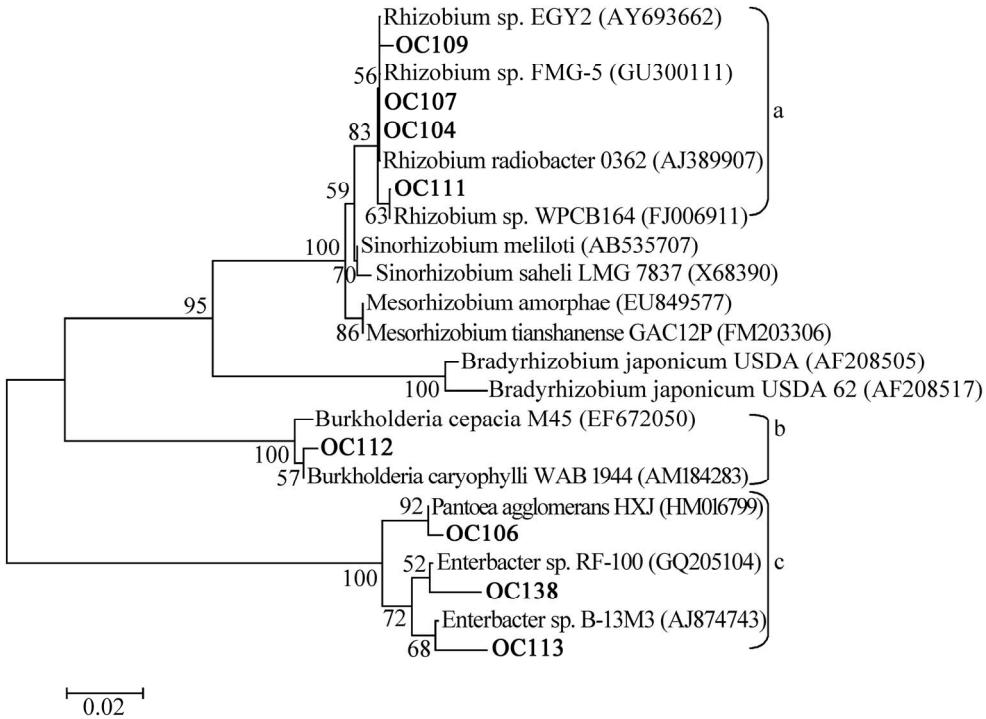

Figure 5. Phylogenetic tree based on the 16S rRNA gene strains of nodule bacteria Onobrychis chorassanica: (a) Alphaproteobacteria; (b) Betaproteobacteria; c- Gammproteobacteria. The branching pattern was produced by the neighbour-joining method. The GenBank accession numbers for the sequences used are indicated in parentheses. Symbionts of Onobrychis chorassanica are shown in bold type.

75-100 cm (**Figure 1**), the plants blossomed and produced seeds thereby providing a renewal of the "green belt". The dry biomass of *Onobrychis transcaucasica* plants reached 323-428 g/m^2, while biomass of *Onobrychis chorassanica* plants was 390-714 g/m^2 in dependence on inoculation with different nodule bacteria strains (**Table 3**). Considerable increase of *Onobrychis transcaucasica* biomass was observed at inoculation of plants with OT121, OT123, OT136 strains. Under cross- and direct inoculation of *Onobrychis chorassanica* plants with OC107, OT118, OT121 strains the highest biomass of plants in comparison with control plants biomass was recorded. It should be noted that under inoculation of plants the numerous nodules on plant roots of both *Onobrychis transcaucasica* (nodule number on each plant exceeded 500 units per 1 plant) and *Onobrychis chorassanica* (more 200 nodules / plant) were detected (**Table 3**, **Figure 6**). During development of root system the plants immobilized significant amount of sand, where *Onobrychis transcaucasica* plants had a fibrous (racemose) root system (**Figure 6**). The measurements of sand accumulation near the foot of the plants showed that if on the edges of the experimental plot there was a layer of sand with 5 cm height, then within internal zone of the plot (in furrows, between rows) the sand layer of 2-3 cm height was detected. By the 2nd year of plants vegetation the sustainable growth of *Onobrychis* plants was recorded (**Figure 7**), where together with adult (mature) plants it was possible to observe an appearance of plenty young sprouts of fallen seeds (seedfall).

Table 3. Sand immobilization with Onobrychis plants (field experiments during 5 months at the Kyzil-Kum Desert Biostastion).

Inoculation variant	Onobrychis transcaucasica		Onobrychis chorassanica	
	Average dry shoot plant biomass, g/m^2	Average height of sand layer at foot of plants, cm	Average dry shoot plant biomass, g/m^2	Average height of sand layer at foot of plants, cm
Control	329 ± 22.0		350 ± 39.0	
OT102	347 ± 34.0		552 ± 30.99	
OT103	361 ± 22.0		409 ± 8.0	
OT115	323 ± 19.0		504 ± 51.0	
OT117	342 ± 24.0		457 ± 20.41	
OT118	400 ± 20.99		695 ±22.99	
OT121	423 ± 40.0	2-3	698 ± 33.0	2-3
OT123	428 ± 27.0		390 ± 25.0	
OT136	428 ± 28.9		433 ± 11.99	
OC104	419 ± 2.0		480 ± 13.0	
OC107	380 ± 34.99		714 ± 14.99	
OC109	390 ± 25.99		438 ± 36.0	
OC111	340 ± 25.0		498 ± 20.99	

Note: Values are the means ± SE, n=2; nodule number on each plant exceeded 500 units per 1 plant *Onobrychis transcaucasica* (*Onobrychis chorassanica* – more 200 nodules / plant), their size varied within range for *Onobrychis transcaucasica* 0.3 – 1.7 cm in diameter (0.3 – 2.2 cm for *Onobrychis chorassanica* plants). Height of *Onobrychis* plants varied within range 75-100 cm and length of roots – 45–65 cm.

(a) (b)

Figure 6. The root system of Onobrychis transcaucasica (a) and Onobrychis chorasanica (b).

Figure 7. The 2nd year, early spring, of vegetation of Onobrychis transcaucasica plants at experimental trial plot (model pasture) at Kyzyl-Kum Desert Biostation (Scientific Center of Plant Production "Botanika", Uzbekistan Academy of Sciences).

Thus, with help of *Onobrychis* plants it is possible to conduct an efficient sand immobilization in a way of their growing on blown sand on condition of necessary irrigation and create artificial pastures in semi-desert conditions in order to increase a productivity of natural pastures. But for this it is necessary to support moisture of sabulous soil (sandy clay) no less than 17% for active vegetation (maximal average moisture of desert sand observed in spring season), blossoming and obtaining of real crop yield of *Onobrychis* plants in conditions of deserted soils.

4. Discussion

Some works devoted to nodulation of leguminous plants with bacteria related to *Bettaproteobacteia* and *Gammaproteobacteria* classes are published lately [19-22]. First L. Moulin with other authors established that bacteria of *Burkholderia* genus [20] formed normal nodules on plant roots. Then other researchers found that isolates of root nodule bacteria from two *Mimosa* species at three sites in Costa Rica belonged to the genera *Burkholderia*, *Cupriavidus*, and *Rhizobium*. Inoculation tests further indicated that both *Cupriavidus* and *Burkholderia* spp. resulted in significantly higher plant growth and nodule nitrogenase activity relative to plant performance with strains of *Rhizobium* [21]. Under identification of bacteria isolated from nodules of *Prosopis juliflora* it was shown that in addition to traditional nodule bacteria the bacteria which had 100% of homology with *Achromobakter xylosoxidans* were found [22]. The repeated inoculation of *Prosopis juliflora* plants with these bacteria led to formation of nitrogen-fixing nodules on plant roots. In the bacteria it was determined an availability of nodC gene that is responsible for nodule formation in legume plants. In other works from the root nodules of the three *Mediterranean* wild legume species *Hedysarum carnosum*, *Hedysarum spinosissimum* subsp. *capitatum* and *Hedysarum pallidum* there were isolated bacteria which belonged to the class *Gammaproteobacteria* and included *Pantoea agglomerans, Enterobacter kobei, Enterobacter cloacae, Leclercia adecarboxylata, Escherichia vulneris,* and *Pseudomonas* sp [23].

In our research devoted to *Onobrychis* plant nodulation we also had heterogeneity of bacteria isolated from nodules. Determination of generic and specific composition of bacterial isolates from nodules of *Onobrychis transcaucasica* and *Onobrychis chorassanica* plants showed that 16S rRNA genes of bacteria were highly identical as to *Alphaproteobacteria*, well as to *Betta-* and *Gammaproteobacteria*. The studied OT102, OT103,

OT111, OT115, OT117, OT121, OT123, OT136, OT139, OT140 bacteria from *Onobrychis transcaucasica* were related to *Rhizobium* genus (*Alphaproteobacteria* / *Rhizobiales* / *Rhizobiaceae* / *Rhizobium*), while OT114, OT148 bacteria were related to *Patnoea* genus and OT124 strain—to *Enterobacter* genus (*Gammaproteobacteria* / *Enterobacteriales* / *Enterobacteriaceae* / *Enterobacter, Pantoea)*. Bacteria from *Onobrychis chorassanica* unlike to bacteria from *Onobrychis transcaucasica* on their 16S rRNA genes were related to three classes of bacteria—*Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria*. OC104, OC107, OC109, OC111 bacteria were related to *Rhizobium* genus, OC112 bacterium—to *Burkholderia* genus (*Betaproteobacteria, Burkholderiales, Burkholderiaceae, Burkholderia*) and OC106, OC118 and OC138 bacteria were related to *Enterobacter* genus. If to consider the origin of nodule bacteria from both *Onobrychis* plant species, then it is possible to notice that the most of studied bacteria are very close neighbours incoming into the same genus of bacteria. But as a whole the nodule bacteria from *Onobrychis* plants comprise a wide scope on phylogenetic tree.

Under studying of nodulation of *Onobrychis* plants it has been established that in microvegetation experiment the shoot part of plants was by 2 times longer than root part and very low nodulation was observed. In vegetation experiment the plant roots developed well and the root length was by 3 times longer than the length of shoot part of plants and practically in all variants the nodules were formed. Proceeding from these results one can suppose that intense growth and root development are one of the main criteria that determines the nodulation in both *Onobrychis transcaucasica* and *Onobrychis chorassanica* plants. The nodule bacteria independently on their belonging to one or another bacterial genus formed full nitrogen-fixing nodules on *Onobrychis* plants. It should be noted that OT103, OT111 and OT117 strains displayed a high efficiency in both *Onobrychis transcaucasica* host plant and *Onobrychis chorasanica* plant too. As our field experiments showed, growing up of inoculated *Onobrychis transcaucasica* and *Onobrychis chorsasanica* plants in sabulous sandy soils in the Kyzil-Kum Desert showed a possibility of sand immobilization with its further stabilization. In addition to stabilization of blown sand it is possible to increase a productivity of semi-deserted by means of creation of artificial renewable pastures—if in natural conditions the productivity of desert pasture comprises usually 100-300 kg/ha (24), then on condition of minimal additional irrigation of *Onobrychis transcaucasica* and *Onobrychis chorassanica* symbiosis in sabulous soils it is possible to increase this productivity up to 30-70 c/ha.

Thus, Onobrychis nitrogen-fixing symbiosis can be used for both increase of biological fertility (restoration) of poorer deserted soils which would promote the desert flora diversity and immobilization of sandy soils with aim to increase of productivity of deserted and semi-deserted pastures under minimal irrigation measures.

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