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Creation of a New Highly Productive Parent Material of Sweet Clover *(Melilotus* Adans.*)* based on Varietal and Microbial Systems

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Abstract: The paper presents the results of studies carried out on the experimental field of Research and Production Center of Grain Farming named after A.I. Barayev Limited Liability Partnership for the creation of new parent material for yellow sweet clover (*Melilotus officinalis* (L.) Pall.) and Volga sweet clover (*Melilotus wolgicus* Poir.) based on varietal and microbial systems. The studies were carried out on southern carbonate chernozem typical for the steppe zone of Northern Kazakhstan according to generally accepted methods. Studies have been conducted to study the nitrogen-fixing activity and productivity of 20 varieties and selection numbers of yellow sweet clover and Volga sweet clover after inoculating the seeds with nodule bacteria *Rhisobium meliloti* strain B1-2013. The authors have identified selection numbers KD-1823, KD-1687, KD-1828 of Volga sweet clover and the KD-1825, KD-1683, KD-1728 of yellow sweet clover, characterized by increased nitrogen fixation of atmospheric nitrogen amounting to 87.9-91.3% (of the total) on inoculated variants and 70.80-88.7% on control variants. These numbers were distinguished by the high productivity of fodder mass and seeds. After a comprehensive assessment of nitrogen fixation, fodder mass productivity and seeds, the best results were observed on two promising selection numbers KD-1687 of the Volga sweet clover and KD-1728 of the yellow sweet clover, which will be used in selection programs in the future when creating varieties of a new generation of sweet clover. According to the results of genetic identification, the following endophytic bacteria were isolated: *Paenibacillus peoriae, Pseudomonas moraviensis, Pseudomonas spp, Pantoea spp, Rahnella aquatica, Bacillus anthracis* and *Bacillus pumilis.*

Keywords: Nodule Bacteria *Rhisobium Meliloti*, Nitrogen Fixation, Yellow Sweet Clover, Volga Sweet Clover, Variety

Introduction

In Kazakhstan, more than 20% of the arable land for the production of fodder grasses is occupied by leguminous grasses, including sweet clover. Sweet clover is a promising fodder, as well as a phytomeliorative, medicinal and honey-bearing crop and more and more attention is paid to it as a green manure crop accumulating a large number of macronutrients after its cultivation (De Dios Guerrero-Rodríguez *et al*., 2011; Posypanov, 1991; Timoshkin and Timoshkina, 2016; Zhumadilova *et al*., 2014). The widespread use of the sweet clover is due to its high environmental plasticity. Due to the deeply penetrating root system, it can grow in a wide range of edaphic conditions; sweet clover is drought-resistant, winter-hardy and with low demand for soil fertility (Luo *et al*., 2016). The greatest productivity of the green mass among perennial legumes is observed in the Volga, white and yellow sweet clover. The white sweet clover is considered to be a more valuable species in terms of fodder value (Kurkin, 2003). Volga sweet clover and yellow sweet clover have high and stable productivity, ensure the production of high-protein fodders and have a positive

effect on all elements of soil fertility (Dridiger, 2014; Zelenin, 2013).

Despite the above, one of the main advantages of the culture, the high efficiency of symbiotic nitrogen fixation, is insufficiently expressed in the existing varieties of sweet clover (Tikhonovich, 2000; Zadorin, 2003). For a long time, the work on studying the problem of plant-microbial symbioses remained a monopoly of microbiological institutes that create highly effective microbial preparations. As a result, the role of the host plant, the most genetically stable partner of the plant-microbial system, in the formation of symbioses was underestimated and targeted selection of legumes for this trait was practically not carried out.

Part of the genes determining symbiosis with nodule bacteria in the sweet clover is involved in controlling the development and functioning of arbuscular endomycorrhiza and the interaction of plants with mycorrhizal fungi and nodule bacteria is considered as a triple symbiotic system (Borisov *et al*., 2007). Therefore, the studies aimed at creating highly productive associations of sweet clover with both endo-symbionts are promising from the point of view of increasing biological nitrogen fixation and symbiotic potential in general, reducing the degree of cost and increasing the ecological orientation of agriculture.

In natural conditions, legumes use only 10-30% of their nitrogen-fixing potential. Inoculation of them with effective selection strains of nodule bacteria increases this indicator to 15-50% and the rest of the reserve can be used to optimize the conditions for the functioning of symbiosis. The genetic nitrogen-fixing potential of legume-rhizobial symbiosis can be significantly increased by coordinated selection of phyto- and rhizobiosymbionts (Tolkachev and Didovich, 2003).

The creation of highly efficient plant-microbial systems in agrocenoses by breeding varieties of sweet clover with high symbiotic potential is an innovative field that opens up opportunities for expanding the adaptive properties of plants, giving them new metabolic functions and, based on this, obtaining high-quality and environmentally friendly agricultural products (Shtark *et al*., 2006).

In this regard, the expansion of research aimed at developing methods for creating and obtaining a new material that combines high productivity with an increased ability to fix atmospheric nitrogen is an urgent task when creating stress-resistant permaculture.

The purpose of our study was to screen varieties and selection numbers of yellow sweet clover and Volga sweet clover based on the study and comparison of varietal and microbial systems.

Materials and Methods

Soil and Climatic Characteristics of the Zone

The study was carried out based on stationary field experiments laid down in the Research and Production Center of Grain Farming named after A.I. Baraev, which is located 70 km from the city of Nur-Sultan, in the chernozem zone of the Akmola region of Northern Kazakhstan.

A characteristic feature of the zone is a flat, sometimes slightly wavy, landscape. The soil at the experimental site is southern carbonate chernozem, with a humus content of 3.0-3.2%, the gross nitrogen content of 0.20-0.26% and gross phosphorus content of 0.10-0.15% in the arable layer. The thickness of the arable layer is 22 cm, the structure is dusty, the reaction of the soil medium is slightly alkaline (pH H_2O 7.0-7.9). The availability of mobile phosphorus and potassium is on average 18 and 420 mg/kg-1, respectively. The absorbed complex is dominated by calcium (up to 80%) and magnesium (11%). According to its properties, the soil is typical for the steppe zone of Northern Kazakhstan.

The climate is characterized as sharply continental, very arid and even dry. The main feature of the climate is the variability of the hydrothermal regime over the years, the average annual precipitation in the study area over 50 years of observations ranged from 318.0-419.2 mm. Of the annual precipitation, 35-40% falls in summer, 15-20% in autumn, 17-25% in winter and 20-25% in spring (data from the Shortandinskaya automated meteorological station). The maximum precipitation (20% of the annual norm) mainly falls in July. The hydrothermal coefficient is 1.4-1.5, in some years 0.6-0.7.

Due to the harsh winter conditions and low spring moisture reserves in the soil, soil and air droughts are often observed. This leads to a significant excess of evaporation over precipitation. Drought several times a decade dries up almost completely the grasses on steppe pastures and hayfields, which leads to unstable fodder production. During the warm period (April to October), the average number of days with atmospheric drought ranges from 23 to 60. The maximum number of days with drought in some years may reach 110-130 or even more. Winter is cold and long with frequent winds and blizzards. The soil freezes to 1.5-2 m and slowly thaws in the spring.

Spring is short, with a rapid spasmodic increase in air temperature and strong winds. Summers are dry and hot, in rare years it is damp and cool. The average temperature of the hottest month (July) is 20.0-23.3°C. On particularly hot days, the air temperature during the day can increase up to 42-44°C and the temperature on the soil surface up to +50 - +60°C. The frost-free period lasts up to 102-130 days, in some years up to 168 days.

During the period of our study, precipitation in March/April was higher than the average annual norm (31.3 mm) by 27.6 mm. However, during the period of active growth and development of sweet clover plants (May/June), there was a shortage of moisture in the soil, while precipitation fell below normal by 60-75%.

The objects of the study were 20 varieties and promising selection numbers of yellow sweet clover (*Melilotus officinalis* (L.) Pall.) and Volga sweet clover *(M. wolgicus* Poir.*)*; including 13 numbers of yellow sweet clover and 7 of Volga sweet clover; and a local strain of nodule bacteria *Rhisobium meliloti* B1-2013, isolated from nodules of sweet clover in the microbiology laboratory of the Center.

The nitrogen-fixing ability of the sweet clover during inoculation with Rhisobium *meliloti* rhizobial bacteria isolated from the nodules of the sweet clover was studied in the field.

Breeding work with the sweet clover was carried out according to the methodological guidelines of the All-Russian Williams Fodder Research Institute (VNII) for the selection of perennial grasses (Smurygin *et al*., 1985). Observations of plants and yield accounting were carried out according to the methodology of the V.R. Williams Fodder VNII (Novoselov *et al*., 1997).

To determine the symbiotic properties of the sweet clover, a nursery of Competitive Variety Testing (CVT) was established to study the nitrogen-fixing ability of varieties and promising numbers of yellow sweet clover and Volga sweet clover. The predecessor is complete steam, agricultural techniques adopted for perennial grasses in the steppe zone: In spring, dust mulching with BIG-3, pre-sowing treatment with rolling before and after sowing. The plot area is 25 m^2, the repetition is fourfold. The nursery is laid out in two variants: With pre-sowing inoculation (treatment) of seeds with bacterial strains and without inoculation (control variant) with a precision seeding drill SSFC-7. The seeding rate of sweet clover seeds is 3-4 million seeds/ha.

Before sowing, inoculation of sweet clover seeds with rhizobial bacteria was carried out (at the rate of 200 mL of inoculant per hectare seeding rate).

To isolate nodule bacteria *Rhisobium meliloti* and endophytic bacteria, the roots of sweet clover plants with nodules were thoroughly washed from soil particles and the nodules were cut off with scissors so as not to damage their tissues. Fresh nodules were cleaned from the soil in sterile water. Then they were immersed for 1 min in ethyl alcohol, then for 3 min in 0.1% aqueous solution of mercuric chloride and to remove the mercuric chloride they were again immersed for 1 min in alcohol. Then they were washed in three cups with sterile water, kept in each cup for 10 min. The nodules were crushed and the pulp was transferred to a Petri dish with the help of a biological loop on a bean agar medium and a bean medium. The pulp was smeared with a spatula on the surface of the medium. Then, with the same spatula, sowing was done for several more cups. The seeded cups were kept in a thermostat at a temperature of 25-27°C. Fast-growing nodule bacteria appear on days 3-4, slow-growing ones appear on days 7-9. The composition of the bean agar medium: Bean broth: 1000 mL; K_2HRO_4: 1g/L, $MgSO_4$: 0.3 g/L, sucrose: 10 g/L, agar-agar: 20 g/L. To prepare a bean broth, 50 g of peas were covered with 1 liter of tap water and boiled for 30-40 min from the moment of boiling. The broth was filtered and brought to 1L with tap water. The composition of the bean medium: bean broth: 1000 mL (100 g of peas per 1 liter of water), sucrose: 20 g/L, K_2HRO_4: 1g/L, agar-agar: 30 g/L. Sterilization at 1.1 atm for 20-30 min, pH before sterilization: 6.6 -6.9, after sterilization: 7.0-7.2 (Netrusov *et al*., 2005).

For the initial build-up of biomass, an inoculant of nodule bacteria of the sweet clover *Rh. meliloti* strain B1-2013 was prepared, which was obtained by seeding bacteria under aseptic conditions on an agarized legume medium of the following composition: K_2HRO_4: 0.5 g; $MgSO_4x7H_2O$: 0.2 g; NaCl: 0.2 g; $CaSO_4$: 0.1 g; yeast water: 100 mL (10 g of yeast kept for 2 days in 100 mL of water at 37°C); $MnSO_4$: 0.005 g; $(NH_4)_6Mo_7O_{24}$: 0.002 g.

To obtain the amount of biomass, the nodule bacteria were then transplanted onto a liquid nutrient medium with a yeast decoction and cultured in depth with aeration at a temperature of 24-26°C.

Molecular genetic identification of endophytic bacteria isolated from nodules of yellow sweet clover and Volga sweet clover was carried out at the National Center of Biotechnology in Nur-Sultan.

Molecular genetic identification of bacteria was carried out by analyzing a fragment of the *16*S rRNA gene. DNA isolation from 24-h bacterial cultures grown on nutrient broth was performed using the Qiagen QIAamp DNA Mini Kit. The PCR reaction was performed with universal primers 8f5'-AgAgTTTgATCCTggCTCAg-3 and 806R-5'ggACTACCAgggTATCTAAT (De Vegas *et al*., 2006). PCR purification of the products was carried out by the enzymatic method using Exonuclease I (Thermo Scientific) and alkaline phosphatase (Applied Biosystems) (Werle *et al*., 1994). The sequencing reaction was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, followed by the separation of fragments on an automatic genetic analyzer 3730xl DNA Analyzer (Applied Biosystems).

The Lasergene computer software package (2DNASTAR, Inc.2, USA) was used to assemble the contigs. For phylogenetic analysis, the Mega X software (Kumar *et al*., 2018) (the Neighbor-Joining (NJ) method) was used. For the strains, 16S rRNA gene

sequences from the Gen-Bank database of nucleotide sequences were used.

To determine the proportion of nitrogen fixed from the atmosphere and used for the formation of the biomass of sweet clover we used the Nitrogen Fixation Coefficient (NFC), as well as the selection of varieties and promising selection numbers of sweet clover with increased nitrogen-fixing ability and the method of comparison with a non-leguminous crop wheatgrass (Posypanov, 1991). The principle of the method is based on the assumption that under identical conditions of cultivation of legumes and grains, the amount of nitrogen obtained from them by the soil is approximately the same.

Determination of the total nitrogen of legumes and grains was carried out by the Kjeldahl method on the UDK-142 device according to State Standard (GOST) 13496.4-93 (fodder, compound fodder, fodder raw materials. Methods for determining the content of nitrogen and crude protein).

Determination of nitrogen, phosphorus and potassium in the soil under the sweet clover crops was carried out according to GOST 26205-91 (Soils. Determination of mobile phosphorus and potassium compounds by the Machigin method in the modification of the Central Research Institute of Agrochemical Services for Agriculture (TSINAO)) and GOST 26951-86. (Soil. Determination of nitrates by ionometric method).

The experimental material was processed statistically according to B. Dospekhov using a personal computer and an AGROS 2.11 and SNEDECOR application software packages (Dospekhov, 1985; Martynov *et al*., 2000; Sorokin, 2004).

Results

Biological fixation of molecular nitrogen of the sweet clover was determined in the field.

The study of the formation of nodules was carried out in the second year of the life of the sweet clover plants.

During the flowering period of various varieties and selection numbers of the sweet clover, 10 plants were selected from each plot in the control variant (without inoculation) and in the variant with inoculation by rhizobial bacteria to account for nodules on plant roots. The formation of nodules on the roots of the sweet clover occurred first on the main root and then on the lateral roots of the corresponding orders, Fig. 1.

When assessing the nitrogen-fixing activity by comparison with a non-leguminous culture, during the period of the study, we identified the selection numbers of yellow and Volga sweet clover with the highest percentage of assimilated nitrogen in inoculated variants compared to the control variant, Table 1.

Of the seven selection numbers of the Volga sweet clover, three numbers were identified with increased content of fixed atmospheric nitrogen in the inoculation variant compared to the control variant: KD-1823, KD-1828 and KD-1687 amounting to 1.8-18.3%. The best selection number KD-1687 absorbed the largest amount of nitrogen (18.3%). Of the 13 selection numbers of yellow sweet clover, five were distinguished with increased content of fixed nitrogen in the inoculation variant compared to the control variant: KD-1825, KD- 1683, KD-1728, KD-1699, KD-1494. The percentage of fixed nitrogen in these numbers was in the range of 0.1-2.6%, the maximum accumulation of nitrogen was noted in the number KD-1825.

Biochemical analysis of the dry mass of the selection numbers of the sweet clover showed that the content of protein, phosphorus and manganese in the inoculated variants was the same or lower than in the control ones. It should be noted that the protein content in the dry mass of yellow and Volga sweet clover was 13.82-23.45% in the inoculated variants and 18.34-23.40% in the control variant and nitrogen content was 2.21-3.75 and 2.93-3.74%, respectively, Table 2.

According to biochemical indicators, the selection numbers KD-1687 of yellow sweet clover and KD-1494 and KD-1683 of Volga sweet clover were distinguished, exceeding the average protein content by 0.6-3.47% and the nitrogen content by 0.1-0.8%.

To assess the effect of pre-sowing inoculation on productivity, the yield of fodder mass and seed mass of the selection numbers of the Volga and yellow sweet clover were taken into account.

Three selection numbers of the Volga sweet clover KD-1823, KD-1687 and KD-1828 inoculated with rhizobial bacteria showed an increase in the yield of green mass by 12.2-18.6 c/ha or by 8.5-13.2% when compared with the control variant; dry matter yield was increased by 2.2-3.5 c/ha or 5.3-9.6%, Table 3. The greatest increase in seed yield from inoculation was observed in the KD-1687 number and amounted to 17.6%, in the KD-1823 and KD-1828 numbers it amounted to 10.5 and 11.1%, respectively.

Of the 13 studied numbers of the yellow sweet clover, the five most productive numbers were: KD-1825, KD-1683, KD-1728, KD-1699, KD-1494, the increase of which in terms of yield of green mass, in comparison with the control variant, was higher than 2.3-14.8 c/ha, in the dry matter by 1.2-4.1 c/ha and in seeds by 0.1- 0.2 c/ha.

The greatest increase in the yield of green mass, dry matter and seeds was observed in the selection number KD-1728 of yellow sweet clover with an increase of 11.7, 9.8 and 13.3%, respectively.

During the flowering period of yellow sweet clover and Volga sweet clover, plants were selected to isolate nodules to identify endophytic bacteria. Molecular genetic analysis has identified various types of endophytic bacteria belonging to different genera. As can be seen from Fig. 2, strain D1 was combined into one cluster with Paenibacillus peoriae during the phylogenetic analysis of

the 16S rRNA fragment of the gene. It is important to note that even when identified in GeneBank, the *16S rRNA* nucleotide sequences of this strain had maximum identity with *Paenibacillus speoriae*.

Strains D7, D6 during phylogenetic analysis were combined into one cluster with *Pseudomonas moraviensis,* which was confirmed by the identification of *16S rRNA* nucleotide sequences in GeneBank, where those strains had maximum identity with *Pseudomonas moraviensis* (Fig. 3).

According to the phylogenetic analysis and identification in GeneBank, strains D4, D5 belong to the species *Pseudomonas sp.* (Fig. 4).

Figure 5, strains D46, D8, D3, D25 were combined into one cluster with *Pantoea sp.* and their maximum identity was also shown when identifying the *16S rRNA* nucleotide sequences of these strains in the Gene Bank.

Strains D39, D29, D17, D14, D11, D9, D10, D13, D16, D19, D28, D38, D12, D15, D18, D30, D34, D40 were identified as *Rahnella aquatic by phylogenetic analysis of the* 16S rRNA *fragment of the gene* (Fig. 6).

As a result of molecular genetic analysis of strains of endophytic bacteria isolated from nodules of yellow sweet clover and Volga sweet clover, carried out by analyzing a fragment of the 16S rRNA gene, we observed the presence of such species as *Paenibacillus peoriae*, Pseudomonas moraviensis, *Pseudomonas sp*., *Pantoea sp*., *Rahnella aquatica*, *Bacillus anthracis* and *Bacillus pumilis* in the nodules.

Table 1: Fixation of atmospheric nitrogen by sweet clover plants inoculated with rhizobial bacteria

Selection number	The average number of nodules, pcs.	Weight of a single plant, g	Nitrogen content in the plant, %	Total assimilated nitrogen, (total nitrogen), mg	Assimilated atmospheric nitrogen, mg	Assimilated nitrogen %
Volga sweet clover (*Melilotus wolgicus* Poir.)						
KD-1823 (control)	9.5	11.86	3.45	411.27	354.52	86.40
KD-1823 (inoculation)	4.5	16.01	2.99	472.75	416.00	88.20
KD-1687 (control)	5.1	6.94	3.28	219.12	162.37	70.80
KD-1687 (inoculation)	9.3	15.54	3.39	521.44	464.69	89.10
KD-1828 (control)	9.0	7.92	3.36	266.80	210.05	77.20
KD-1828 (inoculation)	6.3	11.19	2.71	297.55	240.80	88.50
Yellow sweet clover (*Melilotus officinalis* (L.) Pall.)						
KD-1825 (control)	7.6	9.75	3.74	364.70	323.60	88.70
KD-1825 (inoculation)	19.2	14.10	3.34	470.90	429.80	91.30
KD-1683 (control)	4.0	10.87	3.20	347.80	306.70	88.20
KD-1683 (inoculation)	16.4	11.41	3.75	427.90	386.80	90.40
KD-1728 (control)	7.4	9.20	3.33	306.40	265.30	86.60
KD-1728 (inoculation)	16.2	10.57	3.22	340.40	299.30	87.90
KD-1699 (control)	5.6	9.70	3.58	347.30	306.20	88.20
KD-1699 (inoculation)	23.2	9.78	3.58	350.10	309.00	88.30
KD-1494 (control)	10.0	9.89	3.42	338.20	297.10	87.80
KD-1494 (inoculation)	29.4	8.96	3.52	315.40	274.30	87.00

Table 2: Characteristics of the sweet clover selection numbers distinguished by biochemical parameters, %

Variant	Protein	N	P	K	Ca	Mg
Volga sweet clover (*Melilotus wolgicus* Poir.)						
KD-1823 (control)	20.54	3.29	0.28	1.88	1.37	0.30
KD-1823 (inoculation)	13.82	2.21	0.25	1.43	1.37	0.25
KD-1687 (control)	18.34	2.93	0.28	1.66	1.50	0.28
KD-1687 (inoculation)	19.42	3.11	0.28	2.00	1.35	0.29
KD-1828 (control)	21.38	3.42	0.30	2.05	1.36	0.30
KD-1828 (inoculation)	15.11	2.42	0.25	1.39	1.46	0.26
Yellow sweet clover (*Melilotus officinalis* (L.) Pall.)						
KD-1825 (control)	23.40	3.74	0.28	2.64	1.91	0.37
KD-1825 (inoculation)	20.86	3.34	0.31	1.70	1.27	0.29
KD-1683 (control)	19.98	3.20	0.28	1.78	1.59	0.28
KD-1683 (inoculation)	23.45	3.75	0.33	2.01	1.38	0.31
KD-1728 (control)	20.79	3.33	0.30	1.86	1.67	0.31
KD-1728 (inoculation)	20.11	3.22	0.30	1.69	1.35	0.31
KD-1699 (control)	22.36	3.58	0.29	2.56	1.60	0.35
KD-1699 (inoculation)	22.36	3.58	0.33	1.89	1.17	0.31
KD-1494 (control)	21.43	3.42	0.29	2.51	1.84	0.37
KD-1494 (inoculation)	22.03	3.52	0.31	1.77	1.26	0.30

Table 3: The effect of pre-sowing inoculation on the yield of green mass, dry matter, and seeds from the sweet clover selection numbers

Selection number	Assimilated nitrogen, %	The yield of green mass, c/ha	Increase (± compared to the control variant), c/ha	The yield of dry matter, c/ha	Increase (± compared to the control variant), c/ha	Seed yield, c/ha	Increase (± compared to the control variant), c/ha
Volga sweet clover (*Melilotus wolgicus* Poir.)							
KD-1823 (control)	86.40	142.7	-	41.0	-	1.9	-
KD-1823 (inoculation)	88.50	154.9	+12.2	43.2	+2.2	2.1	+0.2
KD-1687 (control)	70.75	138.6	-	36.4	-	1.7	-
KD-1687 (inoculation)	89.05	156.9	+18.4	39.9	+3.5	2.0	+0.3
KD-1828 (control)	77.20	147.7	-	41.3	-	1.8	-
KD-1828 (inoculation)	80.45	166.2	+18.6	44.4	+3.1	2.0	0.2
Least Significant Difference $(LSD)_{05}$		12.3			4.1	0.1	
Yellow sweet clover (*Melilotus officinalis* (L.) Pall.)							
KD-1825 Control	88.7	111.8	-	-	34.9	1.5	-
KD-1825 (inoculation)	91.3	120.1	+8.3	37.7	+2.8	1.6	+0.1
KD-1683 Control	88.2	110.7	-	-	37.6	1.2	-
KD-1683 (inoculation)	90.4	113.0	+2.3	38.8	+1.2	1.3	+0.1
KD-1728 Control	86.6	126.8	-	39.8	-	1.5	
KD-1728 (inoculation)	87.9	141.6	+14.8	43.7	+3.9	1.7	+0.2
KD-1699 Control	88.2	126.0	-	30.2	-	1.4	-
KD-1699 (inoculation)	88.3	132.8	+6.8	34.3	+4.1	1.6	+0.2
KD-1494 (control)	87.8	112.0	-	36.7	-	1.2	-
KD-1494 (inoculation)	87.0	121.6	+9.6	39.5	+2.8	1.3	+0.1
LSD_{05}		11.6		2.8	0.1		

Fig. 1: Formation of nodules on the roots of the sweet clover

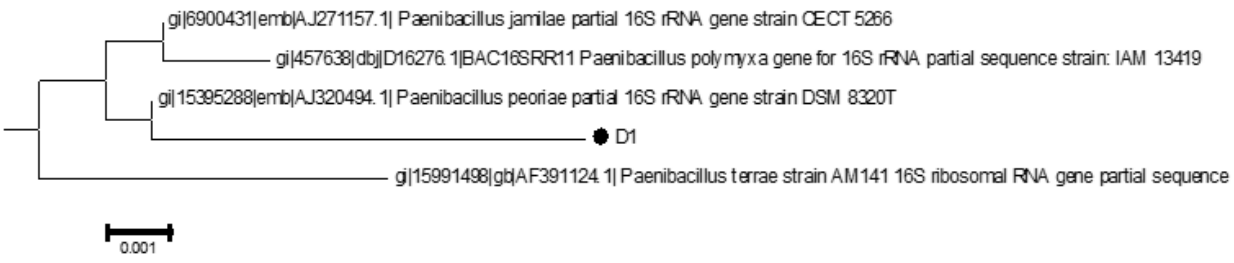

Fig. 2: Phylogenetic tree based on the analysis of a gene fragment *16S rRNA* groups

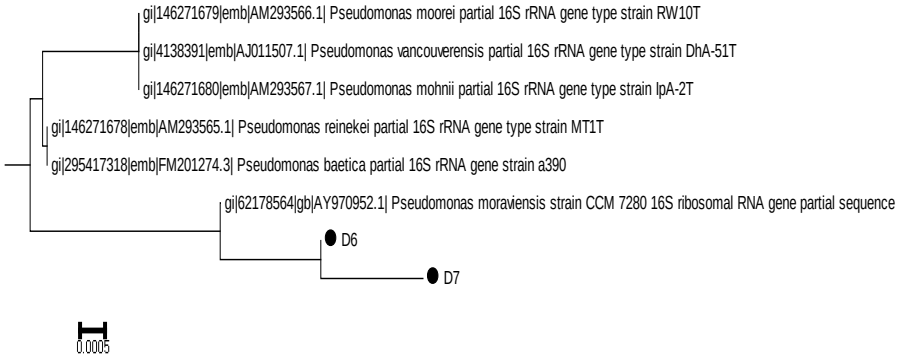

Fig. 3: Phylogenetic tree based on the analysis of a fragment of the *16S rRNA* gene group

Fig. 4: Phylogenetic tree based on the analysis of a fragment of the *16S rRNA* gene group

Fig. 5: Phylogenetic tree based on the analysis of a fragment of the 16S rRNA gene group

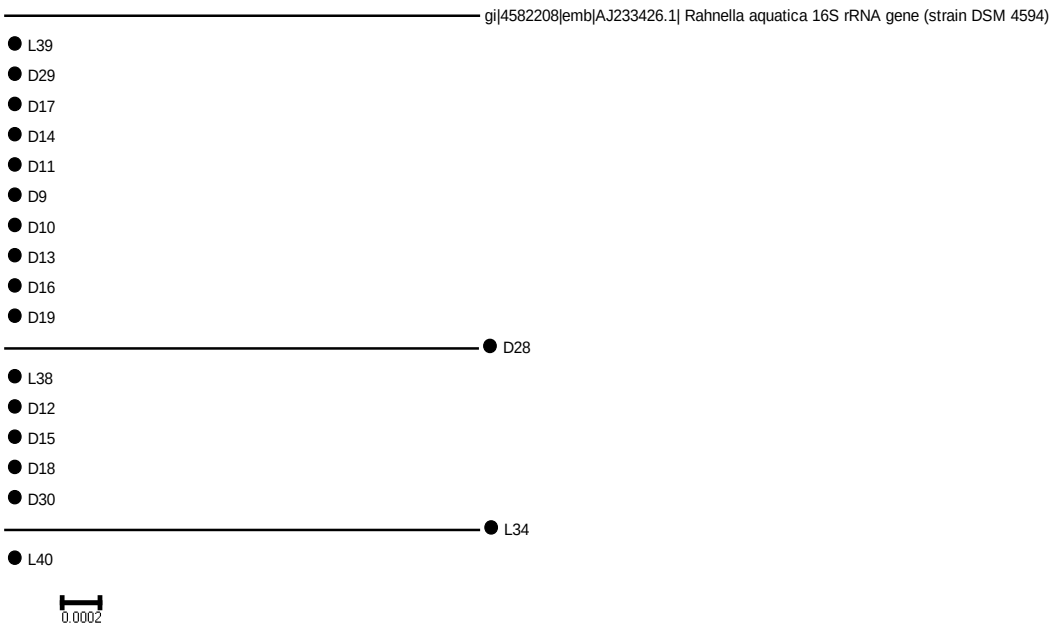

Fig. 6: Phylogenetic tree based on the analysis of a fragment of the 16S rRNA gene group

Discussion

The productivity of the process of symbiotic nitrogen fixation largely depends on the factors that determine the relationship between nodule bacteria and legumes. Nodule bacteria have two main properties concerning legumes: The first is virulence, that is, the ability of bacteria to penetrate the root tissues of the plant, multiply in root nodules and the second is efficiency (activity), i.e., their greater or lesser ability to fix molecular nitrogen in symbiosis with legumes. The effectiveness of nodule bacteria is determined primarily by the genetic nature of this strain (Kretovich *et al*., 2018). In our studies of the B1-2013 strain, *Rhisobium meliloti* played a significant role in the formation of biomass and nitrogen accumulation by sweet clover plants. However, various promising selection numbers did not manifest themselves in the same way concerning this strain. In our studies, this can serve as a basis for creating a new parent material with high nitrogen fixation efficiency. The same conclusions were made by scientists studying the effect of pre-sowing inoculation of alfalfa seeds; it was observed that the Voronezh 6 alfalfa variety had higher responsiveness to seed productivity compared to the Pavlovskaya pestraya variety (Shatskii *et al*., 2010).

The formation of the symbiotic apparatus directly depends not only on the genetic compatibility of the host plant and the rhizobia strain but also on the influence of abiotic factors, including temperature and moisture availability (Egorov, 1989; Khamokov, 2015). The optimal water-air regime for the growth and development of nodules and, in general, legume-rhizobial symbiosis develops at a soil moisture content of 60-80% of the total Field Moisture Capacity (FMC) (Shatskii *et al*., 2010; Stoinier *et al*., 1979). At the same time, the sweet clover can form an effective symbiosis with soil moisture below 60% FMC.

Therefore, we assume that the studied selection numbers have a low level of formation of the number of nodules on the roots of sweet clover plants, associated with a lack of moisture in the soil (35-45% of the FMC). Thus, according to V.S. Worrall and R.J. Roughley (1976), a decrease in soil moisture from 5.5 to 3.5% significantly reduced the number of infectious filaments formed inside the root fibrilla and completely suppressed the nodule formation of *Trifolium subterraneum.*

In addition, the lack of moisture in the soil has a noticeable effect on N_2 fixation, since the nucleation, growth and activity of nodules are more sensitive to water stress than the general metabolism of roots and shoots (Albrecht *et al*., 1984; Zahran and Sprent, 1986).

According to the results of our study, in the selection numbers of the Volga sweet clover, when seeds were inoculated with rhizobial bacteria, on average, only 2.0-9.3 nodules were formed on the roots of plants and 10.0-29.4 nodules were formed on the yellow sweet clover. Nitrogen-fixing activity in 20 varieties and selection numbers of sweet clover inoculated with rhizobial bacteria was quite high and averaged 80%. It is important to note that in addition to the three selected numbers of the sweet clover, the difference in the percentage of absorbed nitrogen between inoculation and control variants was insignificant or negative. This can be observed in the case of using different varieties of legumes, where the productivity of a highly competitive strain directly

depends on the plant variety and an increase in activity on one number can lead to its decrease on another (Dorosinskii, 1970; Shatskii *et al*., 2016).

To determine the productivity of the fodder mass and seeds of varieties and selection numbers of yellow and Volga sweet clover, an assessment was carried out on the yield of green mass, dry matter and seed yield. Our studies have shown that when rhizobial bacteria were treated with sweet clover seeds, there was an increase in the yield of fodder mass and seeds in comparison with the control variant. The greatest increase in the yield of green mass, dry matter and seeds was observed in the numbers KD-1687 of the Volga sweet clover and KD-1728 of yellow sweet clover by 11.7-13.2, 9.6-9.8 and 13.3-17.6%, respectively. Studies conducted in many Russian research institutes to look at the effect of pre-sowing inoculation of legume seeds show an increase in the yield of fodder mass and seeds and demonstrate that symbiotic nitrogen fixation depends on the variety of legume plant and the strain of rhizobial bacteria (Farniev *et al*., 2010; Rumyantseva, 2019; Rumyantseva *et al*., 2019; Shatskii *et al*., 2010, 2016).

Recently, interest in the biodiversity and functions of endophytic bacteria, as well as the prospects for their practical use, has been constantly growing (Berg *et al*., 2008; Clúa *et al*., 2018; Hardoim *et al*., 2015; Jha *et al*., 2013; Pandey *et al*., 2017; Santoyo *et al*., 2016; Vasileva *et al*., 2019). However, the relationship of endophytic bacteria with legumes is of particular interest. Due to symbiotic nitrogen fixation, endophytic bacteria make a significant contribution to maintaining nitrogen balance in agrocenoses. As is known, nodules can be inhabited not only by nodule-forming bacteria but also by various endophytic bacteria typical of the rhizosphere (Dudeja, 2013; Dudeja *et al*., 2012; Ibañez *et al*., 2017).

In legumes, as in other plant species, a rich microbiome of endophytic bacteria is maintained, distributed systemically throughout the body and nodules, as the most nutrient-rich ecological niche of the legume plant, contain the greatest diversity and abundance of endophytic bacteria (Garipova, 2012; Ibañez *et al*., 2017; Martinez-Hidalgo and Hirsch, 2017). In our studies, endophytic bacteria *Paenibacillus speoriae*, *Pseudomonas moraviensis*, *Pseudomonas sp.*, *Pantoea sp.*, *Rahnella aquatica*, *Bacillus anthracis* and *Bacillus pumilis* were identified from the nodules of yellow sweet clover and Volga sweet clover. These bacteria can be considered as potential strains when creating new biological preparations for legumes. For example, the use of *Pseudomonas trivialis* and *Serratia plymuthica* are considered as agents of biological control of the soil-transmitted pathogen *Rhizoctonia solani* (Berg *et al*., 2008).

Conclusion

As a result of the evaluation of 20 varieties and selection numbers of yellow and Volga sweet clover, it was found that the formation of nodules on the roots of sweet clover plants was significantly influenced by the arid conditions of the region during the period of the study.

According to nitrogen-fixing activity, the selection numbers KD-1823, KD-1687, KD-1828 of Volga sweet clover and KD-1825, KD-1683, KD-1728 of yellow sweet clover, were distinguished as the ones having the highest percentage of assimilated atmospheric nitrogen. The selection numbers KD-1823, KD-1687, KD-1828 of the Volga sweet clover and KD-1825, KD-1683, KD-1728 of yellow sweet clover, which have increased nitrogen-fixing activity, were also distinguished by the yield of green mass, dry matter and seeds. The greatest efficiency of the process of biological fixation of atmospheric (molecular) nitrogen was observed in the number KD-1687 of Volga sweet clover and KD-1728 yellow sweet clover, which are characterized by high productivity of fodder mass and seeds.

According to the results of genetic identification, the following endophytic bacteria were isolated from the nodules of yellow sweet clover and Volga sweet clover: *Paenibacillus speoriae, Pseudomonas moraviensis, Pseudomonas spp., Pantoea spp., Rahnella aquatica, Bacillus anthracis* and *Bacillus pumilis*. They can be used in further screening studies of strains capable of fixing atmospheric nitrogen and producing biologically active substances.

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Author's Contributions

All authors equally contributed in this study.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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