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HIGHLY EFFECTIVE ROOT NODULE INOCULANTS OF ALFALFA (*Medicago varia* L.): MOLECULAR-GENETIC ANALYSIS AND PRACTICAL USAGE IN VARIETY CREATION

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Abstract

Among grass stands of perennial forage legume crops, alfalfa is a preferred leguminous plant for the creation of cultivated pastures and restoration of degraded soils. *Medicago sativa* L. nothosubsp. *varia* (Martyn) Arcang is a high-yielding crop and tolerant to adverse cultivation conditions, which is important for risky farming zones in Russia. Alfalfa productivity greatly depends on the success of the formation of a plant-microbial symbiotic system with root nodule bacteria (rhizobia), due to which it becomes able to fix atmospheric nitrogen. Modern symbiogenetics has shown that the effectiveness of symbiotic systems depends on the complementary interactions of the plant and microsymbiont genomes. Proceeding from this, the biological products that are used to treat legume seeds should also contain selected rhizobia strains with corresponding genotypic characteristics. In the present work, a comparative analysis of the grass yield of 73 variety-strain combinations formed by *Sinorhizobium meliloti* isolates from salt affected areas and by two strains 425a and 415b of commercial importance, with alfalfa varieties obtained by classical and symbiotically depended plant breeding approaches was performed. The prospects of selecting highly effective strains which are complementary to economically valuable varieties of alfalfa in model field trials experiments have been shown. The strains A1 and A2 were found to be symbiotically more active with tested alfalfa varieties than strains of commercial importance. It has been evaluated that variety-strain combinations based on varieties obtained by a symbiotically depended plant breeding approach are characterized by increased adaptability, and the potential to increase their yield significantly exceeds 50 %. It was revealed that productivity of variety-strain combinations formed by strain 425a are under the influence of uncontrolled factors as it was shown by two-factor analysis of variance (two-way ANOVA). A high complementarity of strains A1 and A2 to the variety Agnia, and symbiotic characteristics of the strain 425a, led us to study genomic characteristics of these strains. A comparative analysis of genomes done by using DNA biochips approach revealed significant differences between symbiotically highly effective strains. It was established that genes related to symbiotic activity and stress tolerance in strains recovered from salinized soils had mainly a divergent structure. The data of the first stages of the molecular genetic analysis of highly effective strains strongly indicate the need to continue research that will allow targeted selection of microsymbiont strains for modern varieties of alfalfa. Data produced through the research of a number of variety-strain combinations growth in various climatic conditions of the Russian Federation clearly demonstrate the benefit for symbiotically depended plant breeding approach in order to create new economically valuable varieties of legumes claimed by developing a sustainable forage base for farmers.

Keywords: *Medicago varia*, *Sinorhizobium meliloti*, alfalfa varieties, plant breeding approaches, symbiotic genes and stress tolerance genes, DNA biochip SM6kOligo, genomic islands, symbiotically effective variety-strain combinations, two-way ANOVA, sustainable forage base

Formation of sustainable feed supply in the field of livestock and poultry, including the improvement of feed quality, is a prerequisite for effective development of modern agricultural complexes. Cultivation of perennial forage grasses as pasture crops, and inclusion there in cereal and row crop rotation crops can greatly satisfy the meadow farming request in nitrogen and can significantly reduce the ecological risks associated with the use of mineral fertilizers.

Alfalfa is a forage crop providing high-protein feed for livestock and poultry enriched by all necessary vitamins, carbohydrates, mineral salts and microelements, with digestibility reaching up to 70-80 % [1, 2]. Alfalfa has long been cultivated in almost all main soil and climatic areas of Russia. Currently, 125 varieties of alfalfa of three species *Medicago sativa* L. nothosubsp. *varia* (Martyn) Arcang (variegated alfalfa), *Medicago sativa* L. *subsp. sativa* (dark blue alfalfa), and *Medicago sativa* L. subsp. *falcata* (L.) Arcang (yellow alfalfa) are approved for to use [3]. The practical preference is given to variegated alfalfa (hybrid of dark blue and yellow alfalfa), in which high-yielding crop typical for dark blue alfalfa with high quality of dry substance and tolerance to unfavorable cultivating conditions present in yellow alfalfa are successfully combined [1]. Modern winter-hardy varieties of *Medicago varia* could be cultivated in cold humid climate conditions on non-black soils of Russia. *M. varia* is widely used as a green manure to restore soil fertility, including degraded soils. Growing of this forage crop allows accumulation of ecologically safe biological nitrogen in roots and crops residues up to 120-200 kg/ha [4].

Alfalfa nitrogen accumulated in a biologically available form is a result of symbiotic synergy of plants with soil microorganisms – nodule bacteria. It is known that varieties of leguminous plants differ in response to inoculation by strains and even one and the same strain [5-8]. Nodule bacteria (rhizobia) strains used for treatment of plant seeds vary in symbiotic ability with one/several varieties/species of host plants (host specificity) [7, 9]. In many studies aimed to gain yield of leguminous plants, the priority is given to microsymbiont strains [10, 11]. At that, authors note that strains should successfully compete with local strains in rhizoplane of host plant roots both in standard conditions of alfalfa culturing, as well as under the effect of various abiotic factors (aridity, salinity, low or high pH of soil) [8, 12-17]. A number of studies show that strains tolerant to abiotic stress factors more often form effective symbiosis with host plants [15, 18-20]. However, research on selection of plants and microsymbiont strains are carried out independently from each other until present (11, 21), when publications discussing the need for joint studies are also in place [7, 14, 21].

According to the latest symbiogenetic achievements, genetic resources of plants and microorganisms are functionally integrated into one symbiotic system [23]. The fact that legume plant and microbial components and their integration play a key role in formation of symbiotic systems was considered in a number of publications [23-25]. Accordingly, host specificity of strains towards host plant species/varieties should be accounted for at directional obtainment of symbiotic systems, but the number of such studies at present is scarce [7, 21, 26, 27]. In practice, despite the fact that the economic feasibility of using biological products for legumes has been proven [28-32] and for certain types of legumes they use the appropriate biological products, strain-specificity is still not taken into account. As a result of inoculation of crops of different varieties of one type of legume by strain, which is part of the biological product, the formation of "random" variety-strain combinations characterized by significant variation of symbiotrophic indices like plant density, accumulation of plant green mass, protein content (protein nutritional value of the feed), root system development, size and a number of nodules on roots. Finally, it results in reduction of crop yield according to available data [6, 15, 33-35].

It is the principle of complementarity of genomes of legume-rhizobial symbionts that forms the basis of modern biotechnological method for creation of legumes varieties with improved their symbiotrophic indices. For the first time, positive results were obtained at selection of new clover varieties [36]. Works on creation of modern alfalfa varieties, such as Agnia and Taisia [37], were recently initiated. These varieties are already included into the State Register for Selection Achievements and admitted for use in the Russian Federation in 2012 and 2015, accordingly [3]. Due to the fact that new biotechnological approach allows reducing the duration of selection process for 5-7 years it became possible to create legume varieties of new generation with high-yield indices (increase of feed mass and crop yield) faster [37].

Present work resulted in a highly yield alfalfa variety - strain combinations with narrow and wide adaptability based on results of field plot trials carried out within the scope of Geographical Trials Network (GTN) of All-Russia Research Institute of Agricultural Microbiology (ARRIAM). It presents the first data of comparative analysis of complete-genome sequences of two highly effective and one commercial strain (425a) denoting the potential of future genome strain studies aimed at creation of high-yield plant -microbial symbiotic systems.

The purpose of this study was to assess the contributions of alfalfa and microbial components and their interaction to increase yield of symbiotic systems formed by different alfalfa varieties obtained by traditional method of selection and by selection method accounting symbiotrophic indices (joint symbiotic selection method) and highly effective *Sinorhizobium meliloti* strains from ecosystem of Aral Sea region in comparison with commercial strains 425a and 415b used in production of Rhizotorphin for alfalfa.

Techniques. Commercial strains 425a and 415b of *Sinorhizobium meliloti* are used for the bioproduct Rhizotorphin is made (development of ARRIAM); the 141 native strains (isolates) of *S. meliloti* were originally recovered from nodules of wild alfalfa growing on soils with sulfate-chloride type of salinization [38]. Salt tolerance of *S. meliloti* strains was determined by cell growth (OD_{600} = 0.005) in liquid rich medium TY [39] containing 3.5 % NaCl (w/v) during 96 hours at 28 °C and 180 rpm according to [38, 40]. Rm1021 *S. meliloti* strain was used as a reference.

Molecular genetic analysis of strains. Total DNA of bacteria was isolated by using NucleoSpin Tissue Kit (Macherey-Nagel GmbH, Germany). DNA was marked by Bio Prime kit with Klenow fragment of DNA-polymerase I, by accidental octamers and aminoalkyl-dUTP and fluorophores Cy3 and Cy5 (Thermo Fisher Scientific, USA). DNA-samples were cleaned in Microcon columns (Millipore, USA), quantitatively included mark was determined by Nanodrop spectrophotometer (Thermo Fisher Scientific, USA).

Genomes of the strains A1, A2 and 425a were sequenced by MiSeq method (Illumina, USA). From the 7 to the 15 contigs were obtained for each strain. Sequences corresponding to genome islands (GIs) in contigs were searched by using Mauve (http://darlinglab.org/mauve/mauve.html). Presence of

GIs in genomes wass tested by PCR-method according to [41]. DNA-microchips for hybridization done on full genome nucleotide sequence of the reference Rm1021 strain (NC_003037, NC_003047, NC_003078) were provided by the Center for Biotechnology, Bielefeld University, Germany. Each chip contained 6205 gene specific 70-dimensional oligonucleotide probes in three replications. Chips were hybridized according to standard terms [42], scanning was carried out by using Tecan HS 4800 Pro (Tecan, Switzerland), scans were analyzed by programs Genepix (Molecular Devices, USA) and EMMA (Bielefeld University, Germany). Probabilities of gene presence/absence and dynamic cutoff threshold for each trial were calculated by using programs GACK [43] and Statistica 6.0 (Dell Software Company, USA).

Assessment of symbiotic activity of strains. The variety Vega of *M. varia* inoculated by tested strains were grown in tubes 85 cm3 with vermiculite and Krasilnikov-Korenyako medium without nitrogen (standard conditions) or in tubes 60 cm3 with 0.7 % agar with the same medium but with adding 0.6 % NaCl (model salinity conditions) in 8-fold replicas in microvegetative plant tests [13]. Symbiotic effectiveness of strains was assessed by dry mass of inoculated plants (DMP) calculated by aerial-dry mass (each plant sample was dried for 7 days at 37 °C up to constant mass) as regards to mass of non-inoculated plants (Inoculation Free Control, (IFC).

Field plots trials were carried out in ARRIAM Geographical Trials Network (GNT) in four geographical locations on plots with area from 1.8 to 24 m^2 during 2003-2018. In total the 10 varieties of *M. varia* produced by traditional method of selection (further TS) or by joint symbiotic selection method (further JS) and variety Mira of *M. lupulina* (yellow clover) were used for vegetative tests. The varieties Vega 87, Lada, Lugovaya 67, Pastbishchnaya 88, Selena, Marusinskaya 425, Syulinskaya, and Kuzbasskaya were produced by traditional method of selection, whereas Agnia and Taisia, as well as varietal-sample L10/2 were obtained by joint symbiotic selection method. Alfalfa of varieties Marusinskaya 425 (originated by Morshanskaya Selection Station) and Kuzbasskaya (originated by Siberian Federal Research Center of Agrobiotechnologies RAS) were obtained by sampling of wild alfalfa ecotypes, with further selection of plants with high-yield on fertile soil (these varieties included into the State Register for Selection Achievements in 1938 and 1957, respectively) [3]. The variety Syulinskaya adapted to cold climate of central region of Yakutia, it do not include into the State Register. The varieties Vega 87, Lada, and Lugovaya 67 were created for planting on soil with high dosages of mineral fertilizers [3, 44]. The varieties Pastbishchnaya 88 and Selena were obtained by crossing of genotypes with high adaptability and were oriented for planting on weak-acid and moderately cultivated soils [3, 44]. Such varieties were included into the State Register in 1998 and 2000, correspondingly. The varieties Agnia and Taisia were created by using joint symbiotic selection biotechnology for planting on moderately cultured and non-cultured soils (included in the State Register in 2012 and 2015, accordingly [3, 44]. The variety Mira of *M. lupulina* was obtained by chemical mutagenesis of local wild-growing alfalfa plants from Moscow Region [3, 44].

Seeds passed preplanting treatment by powdering or working solution of the preparation; wide-row planting, with row spacing of 0.3-0.7 m, on depth of 2-3 cm; 3-fold or 4-fold replication of plots; seeding rate is 2.4 mil. seeds/ha; fertilizers were not applied (data of GTN) [29]. Statutory reporting on each trial included data of dry matter values (DM; plant mass dried at 105 °C until constant mass) and/or plant green mass (GM) obtained by separate mowing or total values of grass mowing for vegetation period. Experiments on planting of alfalfa varieties could have significantly differed by schemes, but, usually, lasts at least 2-3 years, and number of independent experiments could have varied from 2 to 6. The value of DM and/or GM for each experiment replica and for IFC, as well as the mean value (average) and the errors of the mean value of DM and/or GM were calculated based on the values of DM and/or GM obtained for individual plants of each experiment replica and for IFC.

In addition, the least significant difference (LSD_{05}) showing the significance of differences between the experiment values and the IFC was provided for each field plot experiment [45]. Statistical significance between tested field plots variants and IFC was calculated by Lowest Significant Difference (LSD_{05}; [45]. Upon conduction of experiments with 5-6 years duration with such varieties as Agnia, Taisia, Selena, Marusinskaya 465, Vega 87, Lada, Pastbishchnaya 88 and varietal sample Lugovaya L10/2 the green mass data, mean values, standard error means and LSD_{05} were calculated for each year.

Statistical processing. Yield assessments for variety-strain combinations formed on the basis of the above mentioned alfalfa varieties and *Sinorhizobium* strains were obtained for each plot trial or plant test by evaluation the gain of yield of green (GR) or dry mass of inoculated plants (DPM) or by dry matter (DM), correspondingly, according to the following formula:

$$CY_i = [(PM_i - M_{IFCi})/ M_{IFCi}] \times 100\ \%,$$

where CY_i is crop yield in *i*-th experiment, PM_i is plant mass in *i*-th experiment, M_{IFCi} is IFC weight in the *i*-th experiment according to Geographical Trials Network. Mean crop yield (MCY) was calculated by GM and DM as

$$MCY = (\sum CY_i/n),$$

where *i* is trial serial number, *n* is total number of trials. Outlying yields determined based on relative deviation were ignored at calculation of MCY [45]. MCY error was calculated based on formulae:

$$x = S/\sqrt{n},$$

where *x* is MCY error, *S* is standard deviation calculated for CY values, *n* is total number of trials. For only one CY (result of one trial), error was 5%.

One-way ANOVA was used to assess the validity of dry mass yields of plants inoculated by nodule bacteria strains, at $\alpha = 0.05$ [46]. Two-way ANOVA [46] was carried out for quantitative assessment of contributions of host plant genotypes and genotypes of studied strains to crop yield evaluated by GM or DM.

Correlation coefficient was determined based on description [47], χ^2 with PAST software [48].

Results. Assessment of symbiotic properties of strains in microvegetative plant tests. Upon studying of 141 strains of *S. meliloti* in symbiosis with the variety Vega 87 of *M. varia* under standardized conditions of sterile microvegetative plant tests, we have found the 36 strains forming highly effective symbiosis (mean dispersion of DM yield in inoculated plants was within the range from 77.0 to 157.9 % regarding the reference strain).

The most perspective was given to the six strains (A1-A6) differing by saline tolerance (Table 1). Yields of DMP from inoculation by strains A2, A4 and A5, differed by phenotype, was in averaged 317 %, by strains A3 and A6 (saline sensitive phenotype) — over 180 % in comparison to inoculation free control (IFC). The most yields were established at using of the A1 strain of salt tolerant phenotype in comparison to IFC and reference strain, those were > 500 and 360 %, accordingly (see Table 1). The increase of yield mass in relation to the reference strain was similar in case of A6 strain, exceeding by 70 % in case of A3 strain and was more than twice higher the latter upon inoculation by A2, A4 and A5 strains (see Table 1). Under conditions of model salinization, inoculation of plants by tested strains also promoted the increase of green mass in plants. Statistically valid gain of yields in comparison to reference strain was obtained only in case of A2, A4 and A5 strains (in average over 180 %; $p < 0.05$). An exception was made to A1 strain formed highly effective symbiosis in plant tests under standard conditions, whereas under saline conditions the assessed yield of GM was by more than 20% lower than values obtained for A3 and A6 strains, but similar with data obtained for the reference strain (see Table 1).

1. Symbiotic activity of *Sinorhizobium meliloti* strains with the variety Vega 87 of *Medicago varia* L. in microvegetative plant tests

Strain	P	Standard conditions				Model saline conditions			
		DPM, mg	error MG, mg	DPM, %	error MG, %	DPM, mg	error MG, mg	DPM, %	error MG, %
A1	R	52,5	5,49	508,64	63,71	11,1	1,44	58,4	20,6
A2	S	35,7	2,41	314,23	27,94	17,8	1,41	155,0	20,2
A3	S	28,0	2,87	224,47	33,28	15,2	1,99	118,0	28,5
A4	R	36,0	3,72	317,89	43,17	23,2	1,85	232,7	26,6
A5	S	36,2	3,42	320,19	39,60	20,9	2,66	198,8	38,2
A6	S	21,2	2,50	146,27	28,93	12,6	0,91	81,2	13,1
Rm1021	S	21,2	0,88	146,26	10,26	10,5	0,79	50,4	11,3
IFC		8,63	0,57	0,00	6,58	6,98	0,46	0,0	6,6
LSD_{05}		11,76				4,64			

N o t e . P — phenotype (R — tolerant, S — sensitive to NaCl); DPM — dry mass of inoculated plants (mean value for 10 replications); error MG — error of mean yield of DMP; IFC — inoculation free control; Rm1021 — reference strain; LSD — the least significant difference.

Therefore, analysis of symbiotic properties of the six strains, two of which had salt tolerant, and four were of salt sensitive phenotype, does not yield the relationship between the own salt tolerance of strains and their possibility to form stress tolerant symbiotic systems, that data, does not inconsistent with our previous results [38]. At the same time, analysis of yields of DM obtained under non saline and under saline conditions had revealed a positive correlation: strains, inoculation by which promoted significant increase in crop yield without salinization, also formed highly productive symbiotic systems at saline conditions (correlation coefficient *r* comprised 0.89).

Assessment of symbiotic properties of strains in field plot trials. Symbiotic effectiveness and host specificity of the above strains were tested in symbiosis with 10 varieties of *M. varia* produced by traditional methods of selection or by modern joint symbiotic selection methods, and with the variety Mira of *M. lupulina* (Table 2). Commercial strains 425a and 415b were used as a reference group of strains. A total of 73 variety-strain combinations were tested, of which 32 were tested in 2-6 independent field plots trials within the scope of GTN (see Table 2).

2. Regions of field plots trials with alfalfa varieties (ARRIAM GTN)

Field plot trials	Soil characteristics	Alfalfa (*Medicago varia* L.) varieties
Bryansk Region[1], Bryansk State Agricultural Academy (Bryansk SAA; since 2014 — Bryansk State Agrarian University), 2002-2009	Grey forest weaky-loamy moderately cultured soil (pH 5.7)	Marusinskaya, Selena, Vega 87, Lada, Lugovaya 67
Moscow Region[1], Federal Scientific Center for Feed Production and Agroecology n/a V.R. Williams (FWRC FPA), 2002-2018	Sod-podzol, moderately loamy, weakly cultured soil (pH 4.49-5.08), uncultured soil	Pastbishchnaya, Agnia, Taisia, Selena, Lugovaya 67 (L10/2), Mira (yellow clover *M. lupulina* L.)
Federal State Novgorod Scientific Research and Design Technological Institute of Agriculture (NSRDTIA)[2], 2005-2009, 2018	Sod-podzol, weakly-loamy on clay, moderately cultured soil	Pastbishchnaya, Vega 87, Selena, Lada, Lugovaya 67
"RosAgro" LLC, Volosovskiy District of Leningrad Region[2], 2015	Sod-carbonated cultured soils (pH 6.5)	Agnia, Taisia
Tomsk State Pedagogic University (TSPU)[3], 2002-2006	Grey forest soil	Kuzbasskaya

Continued Table 2

Primorsk R&D Institute of Agriculture (Primorsk NIISH)[4], 2014	Meadow-brown, clay loam	Vega 87
Federal State Budgetary Scientific Institution Yakut R&D Institute of Agriculture n/a M.G. Safonov (Yakut NIISH), State Medical University[4], 2011	Taiga pale-yellow, cryomorphic soils, dark grey sod-podzol moderately loamy, on light-pale clay loam	Syulinskaya

N o t e . [1] — Central, [2] — North-Western, [3] — West-Siberian, [4] — Far East regions of performance of plot trials by Geographic Trials Network (ARRIAM GTN). Soil characteristics, period, and conditions of trials, and varieties of alfalfa are provided subject to reporting cards of GTN (see section Techniques).

3. Symbiotic parameters of plant-microbe symbiotic systems formed by *Sinorhizobium meliloti* strains and *Medicago varia* L. varieties in field plots trials (ARRIAM Geographic Trials Network)

Alfalfa variety	Y	The gain of plant yield after inoculation with strains (to IFC, %)							
		A1	A2	A3	A4	A5	A6	425a	415б
		Central geographic region							
Agnia	1	132.1[3]	163.7[3]	nd	nd	nd	45.0[3]	11.4[3]	33.0[2]
	2	50.5±7.6 172.5[4]	43.5±10.5 109.3[4]	134.9[4]	198.9[4]	nd	nd	35.8[4]	nd
Taisia	1	nd	95.6±0.5	102±10.1	79.3±0.8	99.1±0.9	45.7±0.3	nd	82.3±0.7
	2	nd	62.5±11.4 198.2[9]	nd	nd	nd	nd	36±1.8 143.5[9]	33.0±1.6 87.0[9]
Pastbishchnaya 88	1	nd	nd	nd	nd	10.3[9]	nd	nd	17.1[9]
	2	nd	51.7±2.5	nd	nd	nd	nd	nd	nd
Selena	1	nd	47.4±16.3	nd	nd	nd	nd	nd	nd
	2	nd	115.2±5.8	nd	nd	nd	nd	26.3[1]	nd
Vega 87	1	nd	31.7[3]	nd	nd	nd	nd	nd	nd
Lada	1	nd	36.7[3]	nd	nd	nd	nd	nd	nd
Lugovaya 67	1	nd	6.0[3]	nd	nd	nd	nd	nd	nd
Lugovaya 67 (L10/2)	1	170.3±20.5 78.6[8]	136±14.3 40.2[8]	149±2.6 80.1[8]	171[7] 73.5[8]	80.1[8]	nd	nd	65.4±3.3
	2	133.8[8]	51.0[8]	148.1[8]	nd	nd	nd	nd	50.8[8]
Marusinskaya 425	1	9.9±1.6 27.9[4]	17.0±3.9	17.8±1.2	21.4±0.9	31.7[4]	nd	24.0±4.6	nd
Mira	2	95.7±4.8	nd	nd	nd	nd	nd	nd	nd
		North-Western geographic region							
Agnia	1	nd	nd	nd	nd	nd	163.4[10]	nd	18.3[10]
	2	nd	nd	17.2±7.4	61.8±11.9 142.9[7]	149.3±27.4	143.5±20.1	9.8±0.5	22.9±5.0
Taisia	1	nd	nd	nd	nd	nd	176.6[10]	nd	14.1[10]
	2	nd	nd	nd	29.6[8] 62.8[7]	nd	98.0±7.3 150.0[10]	nd	15.0[10]
Pastbishchnaya 88	1	84.3±4.2	63.0±11.2 8.0[3]	nd	nd	nd	nd	17.1±5.9 39.2[5]	52.0[11]
	2	nd	7.4[3]	nd	nd	nd	nd	22.2[3]	nd
Selena	1	nd	63.7[3]	61.8[13]	25.9[13]	nd	nd	59.6[3]	50.5[13]
	2	nd	63.3[3]	nd	nd	nd	nd	58.3[3]	nd
Vega 87	1	120.1±6.0	81.3±18.3 6.4[5]	44.3[13]	nd	nd	nd	4.0[3]	25.5[13] 133[11]
	2	nd	31.9[3]	nd	nd	nd	nd	5.8[3]	nd
Lada	1	nd	45.4±8.7	nd	nd	nd	nd	27.3[5]	nd
	2	nd	37.3[3]	nd	nd	nd	nd	nd	nd
Lugovaya 67	1	nd	5.9[3]	nd	nd	nd	nd	12.6[3]	nd
	2	nd	6.4[3]	nd	nd	nd	nd	20.5[3]	nd
Mira	1	nd	77±3.9	nd	nd	nd	nd	nd	63±3.2
		West-Siberian geographic region							
Kuzbasskaya	1	nd	14.5[2]	nd	nd	nd	nd	17.3 ± 0.3	nd
		Far East geographic region							
Syulinskaya	1	42.3[9]	48.5[9]	nd	nd	nd	nd	38.1[9]	73.2[9]
Vega 87	1	15.3[6]	nd	nd	18.3[6]	nd	nd	12.2[6]	nd

N o t e. Variety Mira of *Medicago lupulina* L. Y — yield: 1 — green mass of plants (GM), 2 — dry matter (DM). Table provides mean yield gain and standard error of mean gain of plant mass calculated vs. the inoculation free control (IFC) and expressed in percentage (see section Techniques). Years of trials: [1] — 2003, [2] — 2006, [3] — 2007, [4] — 2008, [5] — 2009,([6] — 2011, [7] — 2012, [8] — 2013, [9] — 2014, [10] — 2015, [11] — 2016, [12] — 2017, [13] — 2018; na — no data available.

Since schemes of field plots trials varied (control strains, number of mowing and trials, years of trials, accounted parameters), comparative analysis of symbiotrophic effectiveness of variety-strain combinations formed based on the above-mentioned varieties and nodule bacteria strains was conducted by gain of yields of green mass crops (GM) and/or dry matter (DM), the gain of yields are provided in Table 3. Yields obtained for trials lasting for 2-3-years are provided with indication of the result reporting year (see Table 3).

Assessment of crop yield of the variety Syulinskaya in symbiosis with strain A1 or A2 or with commercial strain 425a did not yield valid differences ($p > 0.05$) between such three variety-strain combinations in conditions of Far East Geographic region (see Table 3). At the same time, the variety Syulinskaya responded to inoculation by commercial strain 415b, since GM yield was approximately 2 times higher than in case of the above-indicated strains (see Table 3). It is interesting to note that statistically valid yields in GM for symbiotic systems of Syulinskaya-A2 and Selena-A2 planted in geographically distant regions (Far East and Central regions, accordingly) were similar (mean value 47.7±9.4 %). Inoculation of the variety Selena by strain A2 promoted 4-fold increase in DM of plants as compared to the use of commercial strain 425a in conditions of the Central region, as symbiotic systems formed by Selena variety with strains A2 or A3 or with commercial strains 425a or 415b did not validly differ by gain of yields in GM in conditions of North-Western geographical region ($p > 0.05$). Yield by DM for combination Selena-A2 was validly higher than for combination Pastbishchnaya 88-A2 (2 times higher) in conditions of the Central region (see Table 3).

High gains of yields (by GM) of the variety Pastbishchnaya 88 was achieved by using strains A1 or A2 in North-Western geographic region (mean value 70.4±8.3 %), that gain was by more than 3 times exceeded mean gain of yields when commercial strain 425a was applied ($p < 0.05$; see Table 3). Herewith, gain of yields by GM obtained for symbiotic systems Selena-A2 and Selena-A3 were similar to yields established for system Pastbishchnaya 88-A2, however by more than 3 times higher than for combination Pastbishchnaya 88-425a. It should be noted at the good response of Pastbishchnaya 88 to inoculation by strain 415b (yields by GM), whilst gain of yields from inoculation by strain 425a could significantly differ by years of trials (2009, see Table 3) in North-Western region. According to findings, symbiotic system Selena-A2 could be recommended for planting in Central and North-Western regions as highly-effective with a wide adaptive potential, and combinations Selena-A3, Pastbishchnaya 88-A1/A2 are — for North-Western region. Also, inoculation of varieties Selena and Pastbishchnaya 88 by commercial strain 415b or 425a could be of interests for last of mentioned geographic region, while the expected gain of crop yields could possibly be significantly lower (see Table 3).

The variety Marusinskaya 425 was tested in symbiosis with the five strains, the variety Kuzbasskaya — with the A2, and both varieties were assessed in symbiosis with commercial strain 425a. Gains of yields for symbiotic systems of the variety Kuzbasskaya with the strain A2 or with commercial strain 425a (the average yields gain was 14.5 and 17.3 %, accordingly, see table 3), and like for Marusinskaya 425 in symbiosis with strain A2 or A3 (mean value 17.9±2.7 %) did not validly differ. Slightly higher gains of yields were registered in case of inoculation of the same variety by strain A4 or commercial strain 425a (see table 3). We should note extremely low symbiotic response of the variety Marusinskaya 425 to inoculation by strain A1, along with gain of yield could have significantly varied by years of trials (2008, see Table 3). The highest gains of yields were obtained for Marusinskaya 425-A5 (yields by GM were in 1.3 times higher than for strain 425a) (see Table 3).

Vega 87 was mainly tested in North-Western regions with strains A1, A2, A3 and with both commercial strains, while with A2 only in the Central region.

Gain of crop yield of combination Vega 87-A2 assessed by GM was in 2.5 times higher in North-Western region than in the Central region, herewith we should note instable yields by years of trials (2009, see Table 3). Higher crop yields were obtained for combination Vega 87-A1 (gain of yield in GM 120.1±6.0 %) and in approximately 2 times lower — for combination Vega 87-A3 in the same geographic region (see Table 3). At the same time, inoculation of the variety Vega 87 by commercial strain 425a did not result in statistically valid gain of yields (4.0±0.2 %; see Table 3). Whereas the usage of commercial strain 415b allowed obtaining gain of mean yield of 25.5 %, the latter meaning could be also increased in few times in conditions of North-Western region depending on year of trials (2016, see Table 3). Combinations Vega-87-A1 and Vega 87-A2 could be considered as high-yield symbiotic systems for North-Western region.

Crop yield of the variety Lada from inoculation by A2 strain in conditions of the Central region, as well as in North-Western region, was similar (gain of mean value was 42.5±2.2 %; see Table 3). Inoculation of plants of the variety Lugovaya 67 was accompanied by formation of effective symbiotic system only when commercial strain 425a was used (DM yield comprised 20.5 %) in conditions of the North-Western region (see Table 3). Accordingly, combination Lada-A2 could be considered as effective with wide adaptability, whereas Lugovaya 67-425a symbiotic system provided statistically valid, but not high gain of yields at planting in the North-Western region.

Therefore, use of commercial strain 425a allows getting gain of crop yields (by GM), varying from 4 to 27.3 % for varieties Vega 87, Kuzbasskaya, Lada, Lugovaya 67, Marusinskaya 425 and Pastbishchnaya 88. Significant yields of ~~in~~ varieties Vega 87 and Pastbishchnaya 88 could be obtained at inoculation by commercial strain 415b. Statistically valid yields of the variety Selena (at least 50 %) was achieved when inoculation was done by any of two mentioned above strains, whereas responsiveness of the variety Syulinskaya to inoculation by strain 415b was in 2 times higher (over 70 %). Tested highly effective strains allowed getting gains of yields by GM from 40 to 80 % for 5 from 8 varieties obtained by traditional selection methods, herewith in case of varieties Pastbishchnaya 88 and Selena strain-varieties combinations were of high adaptive potential.

It should be noted that strain A1 selected as super effective in symbiosis with the variety Vega 87 done in microvegetative plant tests but it also produced the highest gain of yields with this variety in field trials too. Strains A1 and A2 were also tested in symbiosis with yellow clover of the variety Mira. The productivity of the corresponding variety-strain combinations was not inferior but even exceeded the increase in value obtained using commercial strain 415b. Strains A1 and A2 could be considered as perspective for use as a microbial bioproducts for the variety Mira cultivated as siderate on slightly acidic soils.

Thus, the presented results on the symbiotic activity of strains with alfalfa varieties which were produced by traditional selection methods proved that selection of highly effective strains in model plant tests is reasonable and an assessing of the genetic complementarity of micro- and macrosymbionts is important.

Agnia and Taisia are varieties of new generation which were by joint symbiotic selectin method. The productivity of the variety Agnia was evaluated with strain A1, which was used in the process of creating the variety. Crop yield of the variety Agnia was assessed with strain A1 used in process of creation of variety, and with strains A2-A6 in field plots trials in the Central and North-Western regions.

It should be noted the high responsiveness of the variety Agnia for inoculation by strains A1-A4 (average increase in SV of 153.9%; see Table 3) and the variety Taisia towards strains A2-A5 in the Central region. However, the productivity of the same varieties when they were inoculated with strain A6 were comparable to or lower than those when strain 415b was used for inoculation (see Table 3). The variety Agnia was zoned in the Central region, while its planting in other region could provide different gain of yield values. High yields by DM were obtained at inoculating variety Agnia by strains A5 or A6 (average value 146.1%), as well as by strain A4 (more than 60%; see table 3). While strain A6 formed a highly effective symbiosis with the variety Taisia (gain of yields by GM and DM, accordingly 176.6 and 98.0%) in the North-West region. It should be noted that the yield (by DM) of varieties Agnia and Taisia could increase by 2.5—4 times in some years as it was observed in Central region (2008, 2014) and by 1.5—2 times in the North-West region (2012, 2015; see Table 3).

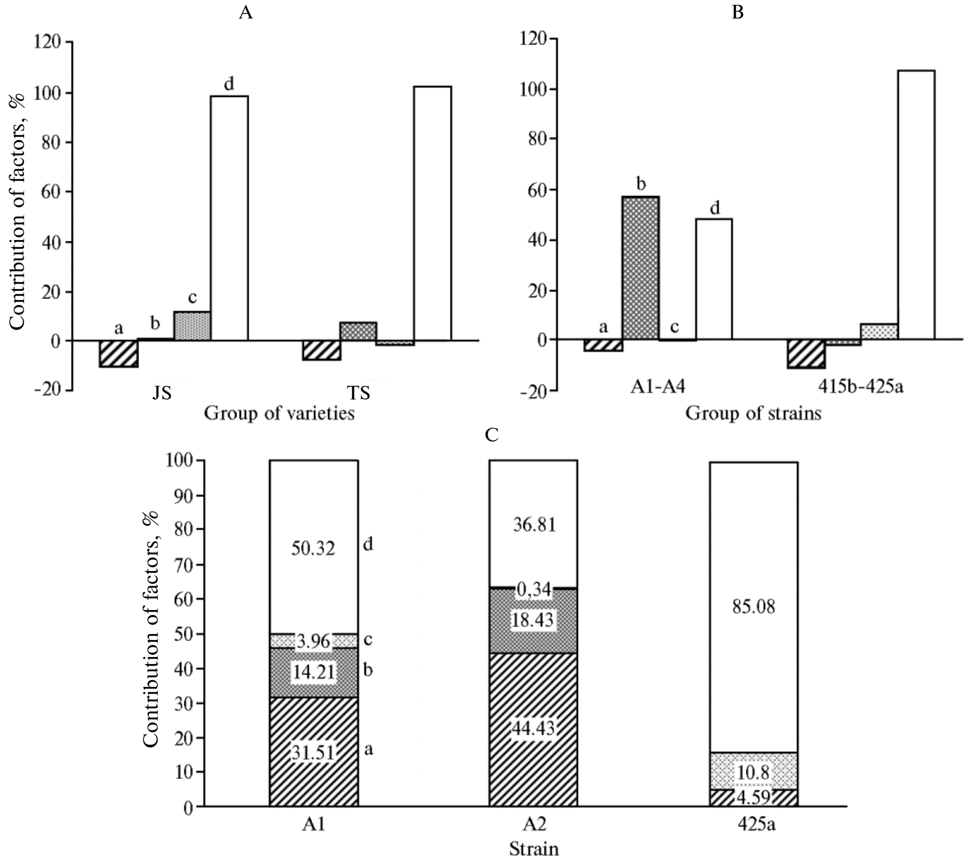
Similar high gains of yields were obtained in experiments with new varietal-sample L10/2 (Stepanova G.V., personal data) inoculated by strains A1-A5. Crop yields by GM for strains A1-A4 were similar and averaged to 154.3±9.0 %, which is 2-fold increased yields obtained when strain 415b was used. In case of strain A2, yield of the varietal-sample L10/2 was in 7 and 23 times higher (depending on year of trial; see Table 3) that yield obtained at inoculation of the original variety Lugovaya 67.

Thus, the increase in DM was no more than 10% and 23% when inoculation of crops of the variety Agnia was done by commercial strains 425a or 415b, respectively, in the North-West region, as well as less than an average of 35% in the case of the variety Taisia inoculated by strain 415b in the Central region. However, in different years, the yield gain was increased by 4.0 and 2.6 times, respectively (2014, see table 3). In case of inoculation of the variety Agnia or varietal-sample L10/2 by strains A1-A4, yields gain varied from 130 to nearly 200 % by years of trials (by DM or by GM). Yields gain in case of variety Taisia inoculated by strains A2-A5 comprised from 79 to 102 % in the Central region (see Table 3).

Two strains A6 and A3 formed highly effective symbiotic systems with varieties Agnia or Taisia in only one of the two geographical regions of the study. The strain A3 formed highly effective symbiosis in the Central region, and strain A6 in the Northwest region. High yield of combination Agnia-A6 was confirmed in commercial trial on plots of 0.1 ha done by "RosAgro" LLC located in Volosovskiy district, Leningrad Region [54]. Gain of crop yield by GM in such trial, comprised 163.4±8.7 % (see Table 3). Strains A4 and A5 (in contrast to the above-mentioned strains) formed high-effective symbiotic systems with both varieties in both geographical regions of study. Summarizing, our findings show that varieties created by joint symbiotic selection method are highly responsible to inoculation, which allows to increase yield 2-3 times due to high specificity of plant-microbe interaction in tested variety-strain combinations.

Two-way ANOVA analysis of plot trial data. Purpose of analysis was to determine the role (contribution) of variability factors from the side of plant symbiont, of microsymbionts, of their variety-strain combinations and of non-controlled factors in formation of crop productivity. An analysis of the gain yield values obtained for varieties Pastbishchnaya 88, Syulinskaya, Vega 87, Marusinskaya 425 (TS varieties) and for Agnia, Taisia, varietal-sample L10/2 (JS varieties) which were inoculated by studied highly effective strains allowed to show that productivity of variety-strain combinations based on JS varieties is largely depended on genetic characteristics of rhizobia strains (contribution value was 11.58%), while the productivity of combinations based on TS varieties, on the contrary, depended on genetic characteristics of corresponding varieties of alfalfa (6.99 %; Fig., A).

Uncontrolled factors in both cases had a similar effect on variety-strain combinations formed based on JS or TS varieties. Assessment of strain contribution had shown that highly effective native strains A1-A4 had actively formed symbiosis on studied alfalfa varieties (contribution was 56.6 %). In case of commercial strains 425a and 415b their genome characteristics had certain contribution (6 %) in gain of productivity of symbiotic systems with considered plant varieties (see Fig. B).



Two-way analysis of crop yield gain of plant-microbe systems formed by alfalfa varieties produced by traditional breeding or by joint symbiotic selection method with highly effective *Sinorhizobium meliloti* strains: A — contributions of varieties derived traditionally and by symbiotically depended selection, in symbiosis with studied highly effective strains; B — contributions of native and commercial strains to crop yield gain formation with studied alfalfa varieties; C — contributions of strains A1, A2 and 425a to crop yield gain formation with studied alfalfa varieties; a — variety-strain combination, b — plant varieties, c — strain, d — uncontrolled factors. JS — alfalfa varieties obtained by joint symbiotic selection (Agnia, Taisia, varietal L10/2); TS — alfalfa varieties obtained by traditional method of selection (Pastbishchnaya 88, Syulinskaya, Vega 87, Marusinskaya 425); A1, A2 and 425a — highly effective strains *S. meliloti*.

The same approach was applied to assess variation factors for formation of crop yields in variety-strain combinations formed by particular strains A1, A2 and 425a and by TS (Pastbishchnaya 88, Syulinskaya, Vega 87, Marusinskaya 425) and JS varieties. As a result, it was found that a significant contribution to productivity of variety-strain combinations was made by both host plant variety and the microsymbiont strain A1 or A2 (31.5 and 44.4% in yield gain, respectively). The contribution of genotype characteristics of host plant varieties to gain crop yield of variety-microbial combinations varied from 14.2 to 18.4 %, accordingly (see Fig., C). It should be noted that the contribution of genotype characteristics of strain A1 on yield gain was 3.96 %. By contrast, analysis of variety-425a combinations showed that uncontrolled factors are main factors by their effect on yield production. The contribution of genotype characteristics of strain 425a was 10.80 % and of host plant was 4.59 % and contribution of both was at least 16 % in crop yield production.

Therefore, we for the first time shown that genotype characteristics of strain 425a more significantly affects formation symbiotic systems, whilst its host specificity regarding to this or other variety is not clear. These findings are giving some explanations why this strain used in bioproducts was so successful in different agrocenosis. At the same time, varieties Agnia, Taisia, and L10-2, created by the joint selection method, showed increased responsiveness to inoculation with strains of nodule bacteria. Further selection of complementary genotypes of plants and microsymbionts will increase their productivity by at least 50%. New variety-microbial systems tolerant to abiotic uncontrolled stress factors will have benefits for various agroclimatic conditions.

Since the high symbiotic complementarity of strains A1 and A2 to the variety Agnia created for planting on moderately cultured and uncultured soils, and the significance of genotypic characteristics of strain 425a was revealed, the study of the genomic characteristics of such highly effective strains became relevant.

M o l e c u l a r g e n e t i c a n a l y s i s o f s t r a i n s. We have conducted comparative analysis of genomes of highly effective strains A1, A2 and 425a by using DNA-biochip SM6kOligo, constructed on the bases of the reference strain Rm1021 [49]. It was established that genome of strain CXM1 (streptomycin-resistant mutant of the strain 425a) [49] lack 508 ORF (open reading frames), and genomes of the strains A1 and A2 lack 601 and 432 ORF, accordingly, while these sequences may have divergent structure. These ORFs, as established, are localized on chromosome, as well as on two megaplasmids, which are typical for *S. meliloti*. A group of 72 ORF, which are important for symbiotic activity was evaluated. The 17 *nod* genes from indicated group are involved in virulence, competitiveness, nodule formation, and host specificity, while other 31 fix and 8 *nif* genes are responsible for nitrogen fixation and effectiveness. All indicated *nod* genes are present in genome of CXM1, whereas sequences of *nod*D3 and *nod*N genes in genome of strain A1 have divergent structure. Analysis done for *fix* genes shown that *fix*L, *fix*T2 and *fix*M in genome of CXM1, *fix*O3, *fix*T2, *fix*P2, *fix*X in genome of strain A1 and, *fix*O3, *fix*L, *fix*N3, *fix*P3, *fix*I2, *fix*H in genome of strain A2 may have divergent structure or had been lost. Similar structural changes were found for *nif*D and *nif*N in genome of strain A1. Analysis of a group of six *nod* genes localized on megaplasmid II of the reference strain Rm1021 revealed structural changes in two genes (SMb20775 and SMb20825) in all tested native strains. Product of SMb20825 is predicted acetyltransferase of NodL family, localized on external cell membrane, which is related to signal receptor of nodule formation process. Blot hybridization of the sequence of six genes localized on chromosome (three *nod*, two *fix* and one *nif* gene) which are important for formation and functioning of symbiosis showed that their structure is conservative in the genomes of all the studied strains.

Besides genes related to symbiosis, the 23 genes responsible for to salt tolerance of rhizobia strains were analyzed. Six groups of genes: *kup*, *kdp*, *trk* and *kef*, responsible for intracellular concentration of potassium cations, *ots/tre* (encoding trehalose accumulation system) and *bet* genes (encoding betaine accumulation, biosynthesis, and catalysis systems) were studied. It was established that four groups of above mentioned genes are present in genomes of all studied strains. Significant structural changes with predicted functional importance were revealed for *bet* and *trk* genes in strains CXM1 and A1, while genes *betB2* and *trkD* are absent (or their sequences are divergent) in CXM1 and A1, correspondingly. Therefore, genes from the considered groups that have divergent sequences (or absent) are most often found in strains adapted to soil saline conditions at which they form a symbiosis with alfalfa plants.

Genomes of strains A1 and A2 and 425a were screened for accessory elements of genome — for genomic islands (GIs), which are phage related sequences participating in horizontal gene transfer [49]. Analysis of contigs obtained for strains A1 and A2 and 425a allowed revealing the six GIs, which presence was confirmed by PCR-detection method (see Techniques section). It was found that, in contrast to the reference strain which harboring the three GIs, in highly effective native strains the three, the two and the one GIs were detected by PCR detection (see Techniques section). Lengths of GIs in the reference strain varied from 19 to 80 kbp, while in highly effective native strains from 10.6 to 44 kbp. Analysis of nucleotide sequences of GIs by sequencing data had shown that one of genomic islands (10.6 kbp) of strain A2 had homology with GI Sme80S (80 kbp) of the reference strain and with genomic island of 10 kbp in strain 425a (82 % homology). The second island of strain A2 is 44 kbp in size was homologous to GI (27 kbp) of strain A1 (85 % homology), while the other two GIs of the same strain did not have homologous sequences between each other and with the third island of the same strain. Presented data designated that GIs revealed in highly effective strains contain various genetic information, that fact confirm GIs participation in horizontal transfer of genes and transfer of new genetic information to recipient strains.

A significant structural and functional differences between gene pools of highly effective strains A1, A2 and commercial strain 425a according to the first data of done molecular genetic analysis are important for the formation of stress tolerant symbiotic plant-microbial systems. Finally, we would like to outline that we have carried out comparative analysis of highly effective strains from Aral Sea region exposed to salinization, and commercial strains 425a and 415b used for bioproduct Rhizotorphin for alfalfa by symbiotic activity and genome characteristics. For the first time it was shown that highly effective strains selected by symbioptophic characteristics in microvegetative laboratory tests are forming productive symbiosis with alfalfa varieties obtained by traditional and by joint symbiotic selection methods. At the same time, plant-microbial systems based on modern varieties are more adaptive and productive (yields gain by over 50 % as compared to varieties of traditional selection). Obtained data denoting that yield gain capacity of variety-microbial systems formed by commercial strain 425a mainly depends on uncontrolled factors (over 80 %). Genomic characteristics of this strain play more significant role in varying the yield increase than combination of this strain with varieties, that, apparently, determines the demand for biological products based on this strain for various alfalfa varieties cultivated in different geographical regions of Russia.

So, the yield (by green mass of plants and dry matter) of plant-microbial systems based on varieties obtained by joint symbiotic selection methods and highly effective strains of root nodule bacteria adapted to salinity significantly exceeded the similar indices of the “randomly formed” symbiotic system based on commercial strains 425a and 415b. Legume varieties of modern selection have increased susceptibility to inoculation by strains of nodule bacteria, and further selection of microsymbionts of complementary genotypes will contribute to a significant increase of yield and the creation of new variety-strain combinations with increased tolerance to abiotic stress factors and expand possibility to cultivate in various agroclimatic conditions. Varieties of modern selection have higher response to inoculation by root nodule bacteria strains, and further selection of strains with complementary genotypes would promote significant increase of crop and creation of new variety-strain combinations with higher tolerance to abiotic stress factors, which could extend possibility of their planting in various agroclimatic conditions. Presented results of the assessment of crop yields gain obtained as a result of field plot trials carried out during many years in Russian regions differing by agroclimatic conditions clearly demonstrate, the need for the widespread introduction of the joint symbiotic selection method in order to create new varieties of alfalfa.

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