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# ESTIMATION OF PHENOTYPIC PRESENTATIONS OF BACTERIAL GENES, CONTROLLING THE EFFICIENCY OF NITROGEN-FIXING SYMBIOSIS WITH PLANTS

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S u m m a r y

**The collection of recombinant species of nodule bacterium of lucerne (*Sinorhizobium meliloti*) with additional copies of dicarboxylic acid transport gene (process, limiting nitrogen fixation) was used and the authors shown, that the symbiosis efficiency is limiting by the plant inability to absolute involvement of biological nitrogen in growth process and the transfer to above-ground organs nitrogen transport forms, accumulation of which in roots and nodules inhibits an energy entry in bacteroides. On the data of factor analysis of vegetative experiment results the amplification succinate permease *dctA* structure gene in complex with specific transcription regulator *dctBD* increases the symbiosis efficiency regardless of plant variety and vegetation conditions, but the amplification of *nifA* and *ntrA* genes (nonspecific regulators of *dctA* gene) required for the increase of $N_2$-fixing activity only at unfavorable for it conditions.**

**Keywords: symbiotic nitrogen fixation, nodule bacteria, leguminous plants, genetic construction, dicarboxylic acids, factorial analysis, symbiotic efficiency, competitiveness, ecologically safe soil tillage.**

Genetic engineering and introduction in plant growing of highly efficient nitrogen-fixing symbiotic bacteria are limited by low reproducibility of the phenotype "increased efficiency of symbiosis", which use to be the result of growing conditions and genotypes of host plants - highly polymorphic cultivars leveling genotypic differences of microsymbionts.

The prospects and constraints for genetic engineering of nitrogen-fixing microsymbionts. Plant symbioses with nitrogen-fixing bacteria have great ecological and agronomic importance as the source of plant-available nitrogen for environmentally sound farming systems and plant growing (1-3). The most effective symbioses are morphologically supported interactions of plants with rhizobia (legumes), actinomycetes *Frankia* (actinorhiza formed by bipartites the group Rosid I) and cyanobacteria *Nostoc* (syncyanoses characteristic for thallophytes, some gymnosperms and flowering plants). Plant symbioses with rhizospheral and endophytic nitrogen-fixing microorganisms (*Azoarcus*, *Azospirillum*, *Flavobacterium*, *Gluconacetobacter*) have great agronomic potential for many cereals and vegetables (4). These symbioses provide mixotrophic nitrogen nutrition in large majority of cultivated crops, though correlation between symbiotrophic and autotrophic types of nitrogen nutrition widely varies depending on plant species and cultivars (5, 6).

Symbiosis of Legumes and Rhizobium is a promising model for genetic and breeding works on improvement the efficiency of symbiotic nitrogen fixation, since it is well studied at molecular, genetic, morphological, physiological and ecological levels (4). Using the methods of mutagenesis, hybridization and genetic engineering, there were obtained rhizobia strains with enhanced symbiotic activity (7-10). However, their practical use is constrained by incomplete manifestation of hereditary factors for symbiosis efficiency associated with loss of symbiotic functions in cultivated plants (5) and low reproducibility of the phenotype "increased efficiency of symbiosis" due to uncontrolled environmental changes and high polymorphism of host cultivar populations.

Design of nodule bacteria (rhizobia) strains with high nitrogen-fixing activity includes activation of gene systems that control key stages of symbiosis and inactivate its negative regulators.

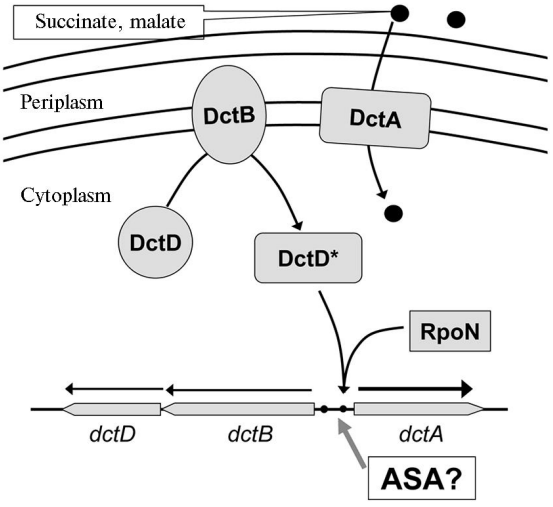


**Fig.1. Symbiotic regulation of *dct*-genes responsible for the transfer of dicarboxylic acids into alfalfa rhizobia (*Sinorhizobium meliloti*):** DctA — succinate permease, DctB and DctD — proteins of two-element regulation system (DctD* is a phosphorylated form of DctD), RpoN — $\sigma^{54}$-subunit of RNA-polymerase, which activates promoters of *nif*-genes; ASA — hypothetical regulator of plant nature involved in activation of the promoter of *dctA*-gene during a symbiosis (18).

Activation of genes encoding symbiotic fixation of nitrogen. Increased symbiotic activity of the rhizobia can be achieved by modification of genes providing signal interaction with a host and nodulation (*nod / nol / noe*) (11), competitive inoculation into plants (*cmp*) (12) and genes encoding nitrogenase and serving its enzymes (*nif / fix*) (13, 14). The efficiency of symbiosis (ES) is usually defined as inoculation effects on plant productivity (1, 15) depending on nitrogen-fixing activity of bacteria and on rhizobia compatibility with homeostasis systems of a plant and its metabolic capabilities. For example, the loss of nitrogen-fixing function was observed in symbiotic bacteria carrying mutant genes for exo- and lipopolysaccharides (suppress protective responses of plants) and enzymes of the Krebs cycle (provide utilization in bacteria of dicarboxylic acids - succinate, malate – supplied by a host plant).

A promising way to enhance nitrogenase activity of bacteria – to improve energy supply of bacteroids with dicarboxylic acids (DA). This transport is controlled by succinate permease DctA, which is synthesized in response to specific regulators DctBD encoded by *dctA*-gene and activating *dctA* transcription at presence of DA, along with non-specific regulators NifA and NtrA involved in activation of nitrogenase synthesis (Fig. 1). Amplification of these genes provides a significant (2-3 fold) rise of nitrogen-fixing activity in vitro (16-18). Although the obtained recombinants can't be immediately introduced in practice owing to their instability in field conditions (19), they can serve as a model for studying genetic and physiological limiting factors of nitrogen fixation.

Penetration of modified rhizobia into plant nodules is another factor important for ES along with activation of nitrogenase system. It depends on

competitiveness (CMP) encoded by multiple *cmp*-genes located in different parts of rhizobia genome including plasmids and chromosomes (13, 20-22). Some *cmp*-genes perform functions important for bacteria survival in soil or in the rhizosphere (growth rate, resistance to antibiotics or phages, molecular structure of cell surface, using soil and rhizosphere sources of nutrition). However, genetic analysis revealed no direct relationship between CMP and nitrogen-fixing activity. Thus, soybean rhizobia (*Bradyrhizobium japonicum*) carrying mutant *nif*-genes and not capable to fix nitrogen demonstrates CMP similar to parental strain (23), while the highly competitive strains of *B. japonicum* serogroup 123 dominant in soils of the USA use to have low or zero nitrogen-fixing activity (24).

The observed data about no correlation between CMP and genetic control of nitrogen-fixing activity indicate the necessity and possibility to combine the factors providing high manifestation degree of these traits in genotypes of commercial rhizobia strains. Many authors described genetic factors suitable for solving this problem. For example, *R. leguminosarum* bv. *viceae* the strain 1-32 was found to carry *Sym*-plasmid (270 kbp) which controls high nitrogen-fixing activity and CMP in contact with spring vetch, along with acid resistance in a liquid medium (25). Rhizobia of alfalfa (*Sinorhizobium meliloti*) possess *nfe*-locus important for manifestation of CMP and located in plasmids (150-200 kbp), so it can be easily transmitted in populations (26).

Expression of several *cmp*-genes was examined after their transfer in rhizobia strains with high nitrogen-fixing activity. A comprehensive investigation was carried out upon the clover rhizobia *tfx*-genes encoding the synthesis of antibiotic trifolitoxine – oligopeptide consisting of 10 amino acids (27). These genes were detected in strain TA1, which forms with clover the inactive (Fix-) symbiosis and at simultaneous inoculation with nitrogen-fixing strains it hinders them from penetration in nodules. Transfer of *tfx*-genes in nitrogen-fixing clover rhizobia strains caused them to raise CMP while trifolitoxine production didn't affect nitrogenase activity. Moreover, transfer of these genes in unrelated species (rhizobia of bean or alfalfa) has led to synthesis of trifolitoxine and resulting increased capacity of these bacteria to compete for inoculation in corresponding plants (27, 28).

However, many *cmp*-genes manifest themselves only in contact with plants and identification of such genes requires direct selection of mutants by CMP trait determined in pot tests. The authors used transposon (Tn5) mutagenesis to obtain a series of alfalfa rhizobia mutants for *cmp*-genes (29), and then transposon-labeled DNA sequences were used to clone the wild-type alleles. Such alleles were transferred into alfalfa rhizobia the strain SKhM1-105 showing high nitrogen-fixing activity, which caused significant raise of its CMP (30).

Inactivation of negative regulators of symbiosis. These factors were first detected in trefoil rhizobia (*Mesorhizobium loti*) on its cryptic plasmid of 240 MDa, removal of which increases both ES and CMP (31). In Tn5-mutants of *Sinorhizobium meliloti* providing weight gain of inoculated alfalfa plants, it has been established a number of hereditary factors negatively affecting symbiosis (32, 33). The primary structure of these regulators (*eff*-genes) was analyzed, and none of them was found to be directly associated with nitrogenase function (33, 34). Some of *eff*-genes control transport of sugars into bacterial cells (34), and inactivation of these genes can increase the rate of DA absorption by bacteria. A similar result is possible after inactivation of genes encoding adenylyl cyclase (35), which relieves catabolite repression limiting utilization of many C-compounds. One of *eff*-genes of *S. meliloti* blocks depolymerization of acidic exopolysaccharides (35), which contributes to overcoming host defense responses preventing rhizobia reproduction in nodules.

Therefore, the analysis of *eff*-genes has shown that ES can be improved by increasing the nitrogen-fixing activity and by optimizing the functions responsible for bacteria compatibility with protective and metabolic systems of host plant.

Physiological constraints of ES exerted by plants. The model showing physiological and genetic factors limiting expression of "increased ES" phenotype were the series of alfalfa rhizobia (*S. meliloti*) recombinant strains carrying extra copies of genes encoding transport of DA - the main energy substrate transferred by the plant into $N_2$-fixing bacteroids (Table 1).

**1. Characteristics of alfalfa rhizobia (*Sinorhizobium meliloti*) recombinant strains obtained by introduction of additional genes encoding transport of dicarboxylic acids into the strain Rm2011 as a part of interactive and replicative vectors**

| Strain | Introduced vector | Copy-number variation (CNV), grade | | | |
|---|---|---|---|---|---|
| | | *dctABD* | *dctA* | *nifA* | *ntrA* |
| 2011-121 | pRmSC121 | 3 | 3 | 1 | 1 |
| 2011-121H6 | pRmSC121H6 | 4 | 4 | 1 | 1 |
| 2011-121SH2 | pRmSC121SH2 | 1 | 3 | 1 | 1 |
| 2011-121HB4 | pRmSC102HB4 | 1 | 1 | 1 | 2 |
| 2011-121HH5 | pWKR56IHH5 | 1 | 1 | 3 | 1 |
| 2011-121/121SH2 | pRmSC121 + pRmSC121SH2 | 3 | 5 | 1 | 1 |
| 2011-H6/SH2 | pRmSC121H6 + pRmSC121SH2 | 4 | 6 | 1 | 1 |
| 2011-SH2/HB4 | pRmSC121SH2 + pRmSC102HB4 | 1 | 3 | 1 | 2 |
| 2011-SH2/HH5 | pRmSC121SH2 + pWKR56IHH5 | 1 | 3 | 3 | 1 |
| Rm2011 (wild-type) | null | 1 | 1 | 1 | 1 |

Note: CNV grade 1 – presence in the wild-type strain of the initial copy of gene, introduction of the copy into integrative or replicative vector – respectively, plus 1 and 3 grades (36).

Testing these strains in sterile pot tests (plants grown on vermiculite with nitrogen-free medium till a flowering stage) revealed in alfalfa cv Du Puits (Table 2) the average gain of nitrogen accumulation in aerial part 4 times exceeding its weight gain (respectively + 69,4 and + 17,9%), while the increase of nitrogen content in plants amounted to 44,1% (36). Thus, most of extra nitrogen fixed by bacteria provides nitrogen enrichment of plant tissues rather than formation of phytomass. The close results were obtained in pea, vetch, mung bean and peanut inoculated with highly active rhizobia strains (37-39).

These facts indicate two processes of legumes' symbiotrophic nitrogen nutrition - enrichment of plants with nitrogen leading to optimized N:C ratio and growth of phytomass. Correlation between these processes is determined by genotypes of symbionts and environmental factors, primarily – availability of fixed nitrogen (4).

**2. Indices of symbiotic activity of *Sinorhizobium meliloti* recombinant strains carrying additional copies of genes encoding transport of dicarboxylic acids, in association with alfalfa the cultivar Du Puits** (pot test) (St.Petersburg – Pushkin, 2002)

| Strain | M | N |
|---|---|---|
| 2011-121[1] | +6,70 | +36,9 (+) |
| 2011-121H6[1] | +49,70 (+) | +87,3 (+) |
| 2011-121SH2[1] | +28,40 | +88,4 (+) |
| 2011-121/121SH2[1] | +39,30 (+) | +126,6 (+) |
| 2011-H6/SH2[1] | +5,10 | +47,5 (+) |
| 2011-121HB4[1] | +37,20 (+) | +83,4 (+) |
| 2011-SH2/HB4[1] | +2,70 | +72,3 (+) |
| 2011-121HH5[1] | –7,20 | +22,9 (+) |
| 2011-SH2/HH5[1] | –0,80 | +59,2 (+) |
| Rm2011 (wild-type)[2] | 59,90 | 910,0 |
| Control (without inoculation)[2] | 32,50 | 315,0 |
| $HCP_{0,05}$[2] | 20,24 | 200,2 |

Note. 1 – deviations (%) from the level in variant of inoculation with the strain Rm2011; 2 — dry aboveground phytomass (M), g/vessel; N – total accumulation of nitrogen in the aboveground phytomass, mg/vessel; (+) – reliable gain.

Therefore, to improve the efficiency of symbiotrophic nitrogen nutrition in plants, it is necessary to determine physiological and genetic factors limiting transformation of fixed nitrogen into extra biomass. It was hypothesized that the delay of fixed nitrogen in roots could be the factor limiting productivity of alfalfa inoculated with *S. meliloti* recombinants. According to L.M. Dorosinskii (1), formation of effective symbiosis in alfalfa, lupine and clover, increases nitrogen content in roots by, respectively, 35, 72 and 44%, in aboveground parts - by 29, 30 and 33%.

**3. Indices of plant productivity, accumulation of nitrogen** (N) **and carbon** (C) **in biomass of alfalfa different cultivars inoculated with *Sinorhizobium meliloti* recombinant strains carrying additional copies of genes encoding transport of dicarboxylic acids** (St.Petersburg – Pushkin, 2002)

| Indicator | Aboveground part | | Root system | |
|---|---|---|---|---|
| | cv Zaykevich | cv Du Puits | cv Zaykevich | cv Du Puits |
| | Deviations from control after inoculation with parental strain Rm2011 (wild-type), % | | | |
| $M_I$ | +31,4 (+) | +36,0 (+) | –41,9 (–) | –61,6 (–) |
| N | +115,2 (+) | +130,0 (+) | –45,5 (–) | –35,5 (–) |
| C | +23,4 (+) | +23,3 (+) | –60,0 (–) | –54,4 (–) |
| | Deviation from the level of strain Rm2011 after inoculation with recombinant strains | | | |
| $M_I$ | +9,5 (+) | +15,3 (+) | –8,5 | +54,3 (+) |
| N | +10,9 (+) | +8,7 | +23,6 (+) | +57,5 (+) |
| C | +1,3 | +14,2 (+) | +9,0 | +53,6 (+) |
| | Total biomass, mg per tube | | | |
| $M_C$ | 10,5 | 10,0 | 8,7 | 11,2 |
| $M_I$ (Rm2011) | 13,8 | 13,6 | 5,1 | 4,3 |

Note. $M_I$ и $M_C$ — respectively, dry biomass in variants with inoculated and control (not inoculated) plants; (+) and (–) — deviations are statistically significant ($P_0 < 0,05$).

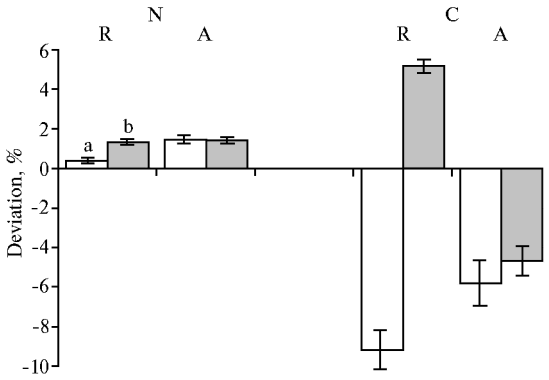


**Fig. 2. The change in contents of nitrogen** (N) **and carbon** (C) **in roots** (R) **and in aboveground part** (A) **of alfalfa cv Zaykevich** (a) **and cv Du Puits** (b) **at symbiosis with *Sinorhizobium meliloti*** (tube test). Average deviations (with standard errors) from control (not inoculated – zero level) are shown for 10 effective strains, see Table 1.

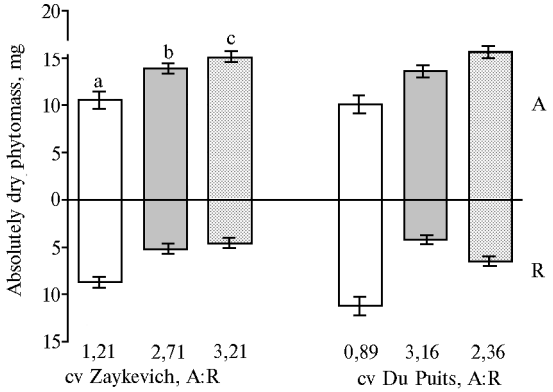


**Fig. 3. The weight ratio of aboveground part** (A) **and roots** (R) **of alfalfa different cultivars during the rise in efficiency of symbiosis with *Sinorhizobium meliloti*** (tube test): a, b and c – respectively, control (without inoculation), inoculation with parental strain Rm2011 (wild-type) and inoculation with recombinant strains. In variants a, b average levels with standard deviations are shown by replicates, in variant c – for a group of 9 strains.

The authors performed tube tests (TT) to study accumulation of biomass, carbon and nitrogen in the roots and aboveground parts of two unrelated alfalfa cultivars - Du Puits (*Medicago sativa*) and Zaykevich (*M. varia*) inoculated with *S. meliloti* the strain Rm2011 and its recombinants carrying extra copies of genes encoding DA transport (35).

It has been found in both cultivars (Table 3) that inoculation with parental strain increases aboveground phytomass along with accumulation in it of N and C, which ratio tended to higher proportion of nitrogen (Fig. 2) owing to intense outflow of carbon into nodules. Inoculation with the strain Rm2011 provided in both varieties a significant decrease in biomass of roots (Table 3), while cv Du Puits demonstrated a more sharp raise of nitrogen content in roots. These differences between alfalfa cultivars were observed after inoculation with recombinants as well: both varieties manifested increased aboveground phytomass and nitrogen content in it (Table 3), but this gain was small and accompanied by accumulation of extra fixed nitrogen in roots – respectively, by 31,0 and 62,0% total nitrogen in cv Zaykevich and Du Puits. At the same time, cv Du Puits showed 5,2% higher carbon content in roots, which was transported from aboveground part and used for assimilation of nitrogen.

In this case, nitrogen content in roots increased by 1,3% corresponding to N:C ratio in amides and amino acids – the transport forms of nitrogen (TN). It is obvious in cv Du Puits the presence of sharp restriction of TN transfer from nodules into aboveground part. On the contrary, cv Zaykevich demonstrates active transport of fixed nitrogen into aerial parts, as it can be seen by the decrease (9,2%) in concentration of TN-bounded carbon in roots. Comparison of these varieties by biomass and nitrogen accumulation reveals significant correlations for the aboveground part ($r$ = +0,61 ... +0,87; $P_0$ = 0,01-0,05), but not for roots. Apparently, genetic differences in ES of these cultivars are determined by peculiarities of biochemical processes in the roots (eg., TN synthesis and transfer from $N_2$-fixing nodule zones into vascular tissue).

The study of plant development indicated some distinctions important for symbiosis, such as weight proportions of aerial parts and roots (A:R): in cv Zaykevich, this ratio grows with rising activity of symbiotic bacteria, in cv Du Puits – it increases during the formation of efficient symbiosis with parental strain Rm2011, but then it drops with increase of ES caused by amplification of the gene encoding DA transport (Fig. 3). Earlier it has been shown in clover species with predominant symbiotrophic nitrogen nutrition (*Trifolium ambiguum*) (5) during the formation of highly effective, low-efficient and ineffective symbiosis with rhizobia – in these variants, A:R ratio equaled to, respectively, 2,21; 1,10 and 0,53 -0,60 (40). In lupine, formation of effective symbiosis with rhizobia increases this ratio from 10,9 to 12,9 (1).

Mathematical assessment of the phenotype "increased ES". Along with incomplete expression of ES genes, practical use of genetically modified rhizobia strains is also limited by high variability of this trait caused by both genetic factors (due to polymorphism in plant population) and environmental conditions. Indeed, legumes cultivars use to be polymorphic populations where quantitative traits of symbiosis vary very widely (41, 42). Two-factor variance analysis of interactions between different genotypes of bacteria and plants revealed in ES the presence of high contribution of uncontrolled variation (usually exceeding impacts of any partner and sometimes – even their cumulative effects) provided mainly by plant population polymorphism (43).

**4. Correlation coefficients of symbiosis efficiency indices in alfalfa different cultivars inoculated with highly-efficient recombinant strains of *Sinorhizobium meliloti* carrying additional copies of genes encoding transport of dicarboxylic acids** (tube tests and pot tests), (St.Petersburg – Pushkin, 2002)

| Indicator | Plants cv Du Puits and cv Zaykevich $TT^1$ | cv Du Puits in different tests | | |
|---|---|---|---|---|
| | | $TT^1$ and $TT^2$ | $TT^1$ and PT | $TT^2$ and PT |
| Dry phytomass, g | +0,49 | +0,65* | –0,53 | –0,29 |
| Nitrogen content, % | +0,61* | –0,05 | +0,02 | –0,25 |
| Total nitrogen content, mg/plant | +0,87** | –0,13 | +0,40 | +0,14 |

Note: $TT^1$ and $TT^2$ – tube tests (36), PT – pot test (see Table 2).
* and ** reliable at $P_0 < 0,05$ и $P_0 < 0,01$, respectively.

Independent studies of the phenotype of recombinants carrying extra copies of genes encoding DA transport showed the low reproducibility of ES values in cv Du Puits (Table 4). Comparing the results of two TT, a reliable correlation was found only for

weight of plants (controlled mainly by varietal genotype). Nitrogen accumulation is known to be determined equally by plant and bacterial genotypes; for this trait, no correlations were found at any comparisons. At the same time, in one of TT of different cultivars (Du Puits, and Zaykevich) a significant coincidence of results was observed – especially for nitrogen accumulation, which trait is controlled by bacterial genotype to greater extent than weight of plant.

To perform the reliable assessment of phenotypes of recombinant symbiotic strains, the authors applied factor analysis, which combines the data of several tests to reveal contributions into ES exerted by individual modified genes (weight of plants, accumulation of nitrogen and carbon) even at low reproducibility of symbiotic phenotype of bacteria (36). In TT with cv Du Puits, simultaneous amplification of the structural gene *dctA* for succinate permease and its specific regulators *dctBD* was found to provide the greater impact on ES indices than amplification of *dctA* gene or its nonspecific regulators *nifA* and *ntrA* (Table 5).

**5. Factor loadings for influence degree caused by amplified copies of genes encoding transport of dicarboxylic acids on efficiency of symbiosis *Sinorhizobium meliloti* with alfalfa different cultivars** (tube tests and pot tests) (St.Petersburg – Pushkin, 2002) (36)

| Gene | cv Du Puits | | | cv Zaykevich |
|---|---|---|---|---|
| | $TT^1$ | $TT^2$ | PT | $TT^2$ |
| *dctABD* | $0,72^a$ | $0,65^a$ | $0,63^a$ | $0,65^a$ |
| *dctA* | $0,39^b$ | $0,38^b$ | $0,65^a$ | $0,58^a$ |
| *nifA* | $0,29^b$ | $0,34^b$ | $0,17^c$ | $0,21^c$ |
| *ntrA* | $0,32^b$ | $0,32^b$ | $0,17^c$ | $0,20^c$ |

Note: $TT^1$ and $TT^2$ – tube tests (36), PT – pot test (see Table 2). The values marked by different letters are reliably significant both in one test and in different tests for one gene.

It can be assumed that insufficient activity of *dctA* gene in TT with cv Du Puits was caused by inhibiting action of TN on DA formation from photosynthesis products (sucrose, glucose) brought into nodules. Against the deficit of DA, activity of specific regulators DctBD is low, and enhanced nitrogen fixation requires amplification of genes encoding non-specific regulators *NifA* and *NtrA*. In cv Zaykevich capable to more active transfer of TN to aboveground organs than cv Du Puits, factor loadings for *dctA* were as high as for *dctABD*, and small – for non-specific regulators *nifA* and *ntrA*. This can be assumed, that rapid removal of TN from nodules typical for cv Zaykevich (Fig. 2) affected high rate of DA formation in nodules providing active work of regulatory system DctBD, therefore, amplification of gene dctA is sufficient for increase in nitrogen-fixing activity.

This assumption was confirmed by trend in distribution of factor loadings for genes encoding DA transport during pot tests (PT) on cv Du Puits and recombinant bacteria – it was similar to cv Zaykevich in TT (Table 5). This similarity can be explained by presence in PT of larger area for plant development than in TT, which allowed higher rates of TN transfer to aboveground part and spending nitrogen on formation extra phytomass.

Previously, inhibitory effect of TN on nitrogenase activity was detected in achlorophillous mutants of pea; these plants showed disturbances of nitrogen-fixing activity in nodules, whose reason wasn't the energy deficit, but accumulation of fixed nitrogen that couldn't be transported to aboveground organs (44). The study of two species of vetch (*Vicia sativa* and *V. villosa*) using isotope ($^{15}N$) methods (45) revealed the reason for low activity of symbiotrophic nitrogen nutrition in *V. sativa* after its domestication and breeding – the increased sensitivity of nitrogenase system to nitrogen compounds formed in nodules during assimilation of fixed nitrogen.

Thus, genetic engineering of highly active nitrogen-fixing symbionts is an important approach for building systems of adaptive farming and plant growing based on environmentally sound and resource-saving technologies. However, there are the factors limiting implementation of this approach: low activity manifested by bacteria owing to plants' inability completely assimilate nitrogen supplied by bacteria and low reproducibility of symbiotic phenotype in bacteria. The first factor relates to physiological limitations for utilization the fixed nitrogen in plants, which can be corrected by genetic modification of plants including the balance of different types of nitrogen nutrition.

In alfalfa, enhanced symbiotrophic nutrition can be achieved by increasing the activity of specific nodule forms of glutamine synthase, glutamate synthase and aspartate aminotransferase – key enzymes in assimilation of fixed nitrogen in plants. The works on breeding legumes for increased activity of these enzymes indicate that primary assimilation is more important for ES than its energy supply (46); when the photosynthesis rate decreased (in mutant plants, after shading), nitrogen fixation in root nodules was blocked owing to excess of TN accumulated in nodules rather than due to the lack of carbohydrates in them (44). However, the presence in alfalfa of clear correlation between nitrogenase and malate dehydrogenase activity suggest that efficient symbiotrophic consumption of nitrogen can be provided by increasing nodules' supply with photosynthesis products (46).

Overcoming of factors limiting manifestation of the trait "increased symbiotic activity" can be done by different approaches. For example, factor analysis of data on pot tests provided an adequate assessment of phenotypic effects from modified transport of DA against highly variable genotypic (polymorphism of cultivars) and environmental background. Reproducibility of best bacterial phenotype can be increased by leveling variability of cultivars' symbiotic properties, which fact is supported by results of tests on alfalfa and fenugreek forms with different levels of polymorphism (41).

These limitations in manifestation of increased symbiotic nitrogen fixation, as well as approaches for overcoming them, must be considered in work on rhizospheral nitrogen fixing bacteria (*Azospirillum, Enterobacter, Flavobacterium*). The authors have shown in barley and wheat cultivars (6) a wide diversity in ratio of symbiotrophic and autotrophic types of nitrogen nutrition, which necessitates breeding work aimed at improved ability of cereals to assimilate biological nitrogen. The data from analysis of plant root exudates performed by All-Russia Research and Development Institute of Agricultural Microbiology (47) suggest the need in selection of plants with increased amount of organic acids excreted by the roots, which is the optimum source of nutrition for symbiotic (rhizobia) and rhizospheral (*Azospirillum*) nitrogen-fixing bacteria.

Thus, design of modified highly active nitrogen-fixing bacteria strains should be combined with creation of plant varieties capable to complete assimilation of biological nitrogen supplied by microsymbionts. Current achievements of molecular genetics allow the optimized work on symbiosis as a complex object of selection, genetic engineering and biotechnology.

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