THE EFFECT OF NUTRIENT SOURCES ON GROWTH OF BRADYRHIZOBIUM JAPONICUM AND AZOSPIRILLUM BRASILENSE AT CO-CULTIVATION

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The paper presents the study of nutrient sources influence on the growth of Bradyrhizobium japonicum and Azospirillum brasilense at their co-cultivation. It was shown that at cultivation of diazotrophs in nutrient media with different concentrations of its components, soybean rhizobia had uneven growth activity. Glucose and molasses was observed to have the greatest impact on the rhizobia count. The results obtained are used as the basis for the optimization of culture medium for co-cultivation of rhizobia and azospirills.

Key words: Bradyrhizobium japonicum, Azospirillum brasilense, growth, nutrients, cultural medium, cultivation.

Beneficial soil microorganisms including rhizobia bacteria used as the agents for different microbial preparations used in modern crop growing technologies play an important role in plant nutrition [1]. The effectiveness of biological preparations highly depends on the titer and functional activity component microorganisms [2]. Thus, it is very important to develop the means that will improve growth activity of diazotrophs ensuring their high nitrogenase activity.

Use of mixed microorganisms' cultures might be one of the ways to enhance the growth of nitrogen fixing bacteria and increase their functional activity. According to our data, and the results obtained by other authors [3, 4] creation of biological preparation for soybeans, which would contain nodule bacteria *Bradyrhizobium japonicum* and associative diazotroph *Azospirillum brasilense* can be very promising. Thus, at co-inoculation of soybeans with these microorganisms the increase of size and weight of individual nodules on the roots of plants as well as nitrogen fixing activity was observed as compared to the pure rhizobia cultures. Co-cultivation of *B. japonicum* and *A. brasilense* also results in higher growth activity of microorganisms, leading to the higher titer of inoculant. At the same time, different nutrient requirements of rhizobia and azospiryls result in number of problems at medium optimization for co-cultivation of diazotrophs.

Based on the foregoing, our research was aimed to study the effect of different nutrient sources on the growth of *Bradyrhizobium japonicum* and *Azospirillum brasilense* in mixed culture.

Materials and methods. Research objects: *Bradyrhizobium japonicum* M-8 [5], *Azospirillum brasilense* 18-2 [6], *Azospirillum brasilense* 410 [7]. All microorganisms were obtained from the National Collection of Useful Soil Microorganisms of Institute of Agricultural Microbiology and AgroIndustrial Production NAAS.

The number of *B. japonicum* was determined using cup method by deep inoculation of suspension in agar medium on pea broth [8]. The number of *A. brasilense* was determined using cup method in agar medium, g/dm³, potato broth – 1, malic acid – 3.6, sucrose – 2.5; pH – 7.2-7.4 (refined with NaOH solution), agar – 11 g.

Petri dishes were kept in an incubator at 28.0 ± 2.0 °C. *B. japonicum* colonies were counted on the tenth day, *A. brasilense* – on the fourth day.

For co-cultivation the medium with dry yeast, glucose, molasses, (NH₄)₂SO₄, KH₂PO₄, K₂HPO₄ · 3H₂O, MgSO₄ · 7H₂O, CaCO₃ was used.

The following variables were studied: (NH₄)₂SO₄ at concentrations of 0.05 g/dm³ to 3.0 g/dm³, KH₂PO₄ – 0.01 g/dm³ to 0.55 g/dm³, K₂HPO₄ · 3H₂O – 0.01 g/dm³ to 0.55 g/dm³, molasses – 1.0 g/dm³ to 45.0 g/dm³, glucose – from 2.0 g/dm³ to 20.0 g/dm³ yeast feed - 1.0 g/dm³ to 10.0 g/dm³, MgSO₄ · 7H₂O – 0.01 g/dm³ to 0.5 g/dm³.

Bacteria were cultivated in 500 cm³ vessels on a shaker with 240 rpm frequency and temperature 28.0 ± 2.0 °C. *B. japonicum* and *A. brasilense* were used at 1:1 ratio and cultivated for 72 hours.

Statistical analysis of the experimental data was performed using Statistica 6.0 software package.

Results. Cultivation of rhizobia in nutrient media with different concentrations of its components, had resulted in uneven growth activity of soybean nodule bacteria. Thus, while determining optimal concentrations of nitrogen sources it was revealed that the highest growth activity of *B. japonicum* M-8 was observed at cultivation of microorganisms in the nutrient medium with a mass fraction of (NH₄)₂SO₄ – 1.0 g/dm³ (Table 1). Reduction of the mass fraction diammonium sulfate in the nutrient medium had led to the decrease of *B. japonicum* number. C0-cultivation of rhizobia with *A. brasilense* 18-2 in the medium with (NH₄)₂SO₄ as the nitrogen source with a mass fraction of 0.5 g/dm³, 0.1 g/dm³, 0.05 g/dm³ the number of *B. japonicum* M-8 was lower, respectively by 16%, 68% and 92% compared to the highest obtained number of rhizobia in the experiment. The similar effect on the growth of rhizobia was observed using smaller content of diammonium sulfate co-cultured with *A. brasilense* 410: the number of *B. japonicum* had decreased by 14%, 20% and 51% respectively compared to the highest obtained number of these bacteria.

Co-cultivation of *B. japonicum* M-8 with *A. brasilense* 18-2 in the nutrient medium with high content of nitrogen had resulted in reduction of the rhizobia number by 8% (at 1.5 g/dm³ and 2.0 g/dm³) and 32% (at 3.0 g/dm³). Joint cultivation of *B. japonicum* M-8 with *A. brasilense* 410, at higher content of diammonium sulfate the reduction trend of rhizobia number reduction was observed.

While determining the effect of optimal concentration of phosphorus source for microorganisms nutrition it was noticed that the highest growth activity of *B. japonicum* M-8 was observed at their co-cultivation in culture medium with a mass fraction of KH₂PO₄ – 0.15 g/dm³, 0.25 g/dm³ and 0.35 g/dm³ (Table 2).

Table 1. Effect of nitrogen source on the growth of *B. japonicum* and *A. brasilense* in liquid nutrient media upon their co-cultivation

Concentrations (NH ₄) ₂ SO ₄ , g/dm ³	Number of bacteria, billion cells / cm ³			
	<i>B. japonicum</i> M-8	<i>A. brasilense</i> 18-2	<i>B. japonicum</i> M-8	<i>A. brasilense</i> 410
0,05	0,3 ± 0,12	2,7 ± 0,30	1,7 ± 0,15	2,7 ± 0,15
0,1	1,2 ± 0,20	3,5 ± 0,12	2,8 ± 0,18	3,0 ± 0,12
0,5	3,1 ± 0,20	3,6 ± 0,12	3,0 ± 0,15	3,3 ± 0,12
1,0	3,7 ± 0,15*	3,8 ± 0,15	3,5 ± 0,18	3,7 ± 0,15
1,5	3,4 ± 0,12	3,6 ± 0,15	3,4 ± 0,21	3,5 ± 0,15
2,0	3,4 ± 0,20	3,4 ± 0,15	3,3 ± 0,15	3,4 ± 0,15
3,0	2,5 ± 0,20	3,4 ± 0,15	3,2 ± 0,15	2,7 ± 0,15

*Note. The highest number of bacteria (within the limits of experimental error) is highlighted in bold

The data obtained had showed that the highest number of rhizobia were observed at KH₂PO₄ concentrations 0.15 g/dm³ and 0.25 g/dm³, but upon the increase of salt mass fraction to 0.45 g/dm³ the titer of *B. japonicum* M-8 cultured together with *A. brasilense* 18-2, was reduced by 14%.

The highest titer of *B. japonicum* M-8, co-cultivated with *A. brasilense* 410 was seen in the medium containing KH₂PO₄ – 0.05 g/dm³, 0.15 g/dm³ and 0.25 g/dm³. Decrease of KH₂PO₄ mass fraction to 0.01 g/dm³ had resulted in reduction of rhizobia number by 26%, while increasing of its mass fraction to 0.35 g/dm³, 0.45 g/dm³ and 0.55 g/dm³ the studied index had decreased, respectively, by 14%, 26% and 34%. Presented data had indicated that co-cultivation of *B. japonicum* M-8 with azospirills, the recommended dose of KH₂PO₄ is 0.15 g/dm³.

Efficiency study of different K₂HPO₄ × 3H₂O concentrations had showed that the highest titer of *B. japonicum* M-8 was observed within the 0.15 g/dm³ and 0.25 g/dm³ of dipotassium hydrogen. Both increase or decrease in the content of K₂HPO₄ × 3H₂O in the medium, had significantly reduced the titer of rhizobia.

Molasses and glucose were used for determination of the optimum mass fractions of carbon sources in the culture medium. The highest growth activity of *B. japonicum* M-8, co-cultured with *A. brasilense* 18-2 was observed in variants with 10.0 g/dm³, 15.0 g/dm³ and 20.0 g/dm³ of molasses (Table 3). At this, the rhizobia titer had ranged from 3.6 billion cells / cm³ to 3.7 billion cells / cm³.

Table 2. Effect of phosphorus sources on the growth of *B. japonicum* and *A. brasilense* in liquid nutrient medium at their co-cultivation

Phosphorous concentrations in phosphates form, g/dm ³	Number of bacteria, billion cells / cm ³			
	<i>B. japonicum</i> M-8	<i>A. brasilense</i> 18-2	<i>B. japonicum</i> M-8	<i>A. brasilense</i> 410
KH₂PO₄				
0,01	0,8 ± 0,12	2,2 ± 0,18	2,6 ± 0,23	2,6 ± 0,20
0,05	1,9 ± 0,18	3,5 ± 0,09	3,4 ± 0,09	3,3 ± 0,09
0,15	3,7 ± 0,03	3,7 ± 0,12	3,5 ± 0,25	3,6 ± 0,23
0,25	3,7 ± 0,15	3,8 ± 0,15	3,5 ± 0,18	3,7 ± 0,15
0,35	3,5 ± 0,15	3,7 ± 0,15	3,0 ± 0,09	3,5 ± 0,18
0,45	3,2 ± 0,12	3,6 ± 0,15	2,6 ± 0,15	3,0 ± 0,15
0,55	2,7 ± 0,12	3,3 ± 0,22	2,3 ± 0,12	3,0 ± 0,24
K₂HPO₄×3H₂O				
0,01	0,7 ± 0,15	1,7 ± 0,24	1,8 ± 0,12	2,4 ± 0,18
0,05	1,6 ± 0,18	3,5 ± 0,15	2,7 ± 0,35	3,1 ± 0,12
0,15	3,7 ± 0,07	3,6 ± 0,18	3,4 ± 0,26	3,5 ± 0,18
0,25	3,7 ± 0,15	3,8 ± 0,15	3,5 ± 0,18	3,7 ± 0,15
0,35	3,5 ± 0,15	3,7 ± 0,12	3,3 ± 0,15	3,6 ± 0,15
0,45	3,3 ± 0,15	3,7 ± 0,15	2,8 ± 0,12	3,3 ± 0,12
0,55	2,0 ± 0,09	3,5 ± 0,17	2,5 ± 0,15	2,7 ± 0,17

Molasses concentration range for *B. japonicum* M-8 co-cultured with *A. brasilense* 410 was broad – from 10.0 g/dm³ to 25.0 g/dm³, while rhizobia titer had ranged from 3.2 billion cells / cm³ to 3.5 billion cells / cm³. Either decrease or increase the mass fraction of molasses in the medium, the number of *B. japonicum* M-8 had significantly decreased. For all investigated molasses concentrations, the 10.0 g/dm³ is recommended for rhizobia co-cultivation with azospirills.

Table 3. Effect of carbon source on the growth of *B. japonicum* and *A. brasilense* in liquid nutrient medium at their co-cultivation

Concentration of carbon source, g/dm ³	Number of bacteria, billion cells / cm ³			
	<i>B. japonicum</i> M-8	<i>A. brasilense</i> 18-2	<i>B. japonicum</i> M-8	<i>A. brasilense</i> 410
Molasses				
1,0	2,6 ± 0,17	0,8 ± 0,15	2,6 ± 0,20	2,2 ± 0,20
5,0	3,0 ± 0,15	2,9 ± 0,12	3,1 ± 0,12	3,0 ± 0,09
10,0	3,7 ± 0,15	3,8 ± 0,15	3,5 ± 0,18	3,7 ± 0,15
15,0	3,6 ± 0,07	3,7 ± 0,18	3,4 ± 0,18	3,8 ± 0,15
20,0	3,6 ± 0,12	3,7 ± 0,12	3,4 ± 0,15	3,6 ± 0,17
25,0	3,2 ± 0,15	3,7 ± 0,23	3,2 ± 0,15	3,4 ± 0,09
30,0	3,3 ± 0,12	3,6 ± 0,15	2,7 ± 0,15	3,1 ± 0,12
35,0	2,6 ± 0,15	3,4 ± 0,09	2,2 ± 0,12	2,3 ± 0,12
40,0	2,3 ± 0,15	2,7 ± 0,12	2,3 ± 0,15	2,0 ± 0,19
45,0	1,2 ± 0,24	2,1 ± 0,21	1,8 ± 0,17	2,7 ± 0,15
Glucose				
2,0	1,7 ± 0,15	3,4 ± 0,23	2,1 ± 0,09	3,4 ± 0,15
4,0	2,8 ± 0,24	3,4 ± 0,21	2,5 ± 0,15	3,4 ± 0,12
6,0	3,3 ± 0,18	3,5 ± 0,20	3,1 ± 0,12	3,5 ± 0,12
8,0	3,5 ± 0,12	3,4 ± 0,20	3,4 ± 0,12	3,5 ± 0,15
10,0	3,8 ± 0,09	3,5 ± 0,12	3,5 ± 0,12	3,6 ± 0,12
12,0	3,7 ± 0,15	3,5 ± 0,21	3,5 ± 0,18	3,7 ± 0,15
14,0	3,7 ± 0,09	3,3 ± 0,15	3,5 ± 0,20	3,5 ± 0,17
16,0	3,7 ± 0,22	3,5 ± 0,09	3,6 ± 0,15	3,3 ± 0,18
18,0	3,9 ± 0,09	3,3 ± 0,09	3,5 ± 0,15	3,1 ± 0,17
20,0	3,9 ± 0,12	3,2 ± 0,12	3,4 ± 0,15	3,1 ± 0,21

Rhizobia cultivation in medium with glucose concentrations ranging from 10.0 g/dm³ to 20.0 g/dm³, the number of *B. japonicum* M-8 was at the level of 3.7 billion cells / cm³ to 3.9 billion cells / cm³ at co-cultivation *A. brasilense* 18-2 and from 3.4 billion cells / cm³ to 3.6 billion cells / cm³ – with *A. brasilense* 410. Reduction of glucose content in medium, the number of soybean rhizobia had also reduced. Glucose concentration at 10.0 g/dm³, is recommended. The increasing of glucose content in the medium is economically unreasonable as the changes of bacteria number was within the error.

As it was showed in our previous studies it was recommended the use of dry yeasts for co-cultivation of *B. japonicum* M-8 with *A. brasilense*. Their use is determined by the fact that yeast is rich in proteins as their content can reach 66%, with 10% of essential amino acids. In addition, yeasts is also a source of growth factors, including vitamins.

Our studies indicate that all investigated concentrations were effective except of 1.0 g/dm³ and 10.0 g/dm³ (Table 4) at co-cultivation of rhizobia with *A. brasilense* 18-2.

Co-cultivation of *B. japonicum* M-8 with *A. brasilense* 410 in medium with glucose concentrations ranging from 3.0 g/dm³ to 8.0 g/dm³ had not affected the number of rhizobia, which have stayed within the error. Thus, the economically justified dose of dry yeast was 3 g/dm³.

Magnesium is required by rhizobia in small amounts for maintenance of bacteria structure and functioning of enzyme systems in microorganism cells. Magnesium – is an essential component of the enzyme ATP-dependent biochemical reactions. It is involved in the metabolism of carbohydrates, proteins and fats, as well as redox reactions. Magnesium activates glycolytic enzymes, enzymes cellular oxidation enzymes synthesis of nucleic acids. Effect of different concentrations of MgSO₄ × 7H₂O in nutrient medium on growth activity of diazotrophs was also studied.

Table 4. Effect of dry yeasts on growth of *B. japonicum* and *A. brasilense* in liquid nutrient medium at their co-cultivation

Concentrations, g/dm ³	Number of bacteria, billion cells / cm ³			
	<i>B. japonicum</i> M-8	<i>A. brasilense</i> 18-2	<i>B. japonicum</i> M-8	<i>A. brasilense</i> 410
1,0	3,5 ± 0,15	3,4 ± 0,15	2,7 ± 0,15	3,2 ± 0,12
2,0	3,8 ± 0,12	3,5 ± 0,20	3,3 ± 0,09	3,4 ± 0,15
3,0	3,6 ± 0,21	3,6 ± 0,18	3,4 ± 0,08	3,5 ± 0,12
4,0	3,7 ± 0,23	3,3 ± 0,18	3,6 ± 0,12	3,5 ± 0,17
5,0	3,7 ± 0,15	3,5 ± 0,21	3,5 ± 0,18	3,7 ± 0,15
6,0	3,7 ± 0,20	3,5 ± 0,12	3,5 ± 0,27	3,3 ± 0,15
7,0	3,6 ± 0,22	3,5 ± 0,21	3,5 ± 0,12	3,1 ± 0,15
8,0	3,6 ± 0,15	3,3 ± 0,21	3,4 ± 0,20	3,1 ± 0,23
9,0	3,7 ± 0,20	3,3 ± 0,17	3,3 ± 0,17	2,8 ± 0,15
10,0	3,3 ± 0,15	3,4 ± 0,18	3,3 ± 0,15	2,6 ± 0,15

The data presented in Table 5 indicate that the highest number of *B. japonicum* M-8 co-cultivated with *A. brasilense* 18-2 was observed in variants with $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ concentration ranging from 0.1 g/dm³ to 0.5 g/dm³.

Table 5. Effect of $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ on growth of *B. japonicum* and *A. brasilense* in liquid nutrient medium at their co-cultivation

Concentrations, g/dm ³	Number of bacteria, billion cells / cm ³			
	<i>B. japonicum</i> M-8	<i>A. brasilense</i> 18-2	<i>B. japonicum</i> M-8	<i>A. brasilense</i> 410
0,01	2,8 ± 0,15	3,3 ± 0,15	2,7 ± 0,17	2,8 ± 0,24
0,05	2,9 ± 0,12	3,6 ± 0, 21	2,6 ± 0,18	3,4 ± 0,17
0,1	3,6 ± 0,15	3,4 ± 0,23	3,3 ± 0,12	3,6 ± 0,09
0,2	3,5 ± 0,20	3,6 ± 0,20	3,5 ± 0,18	3,7 ± 0,15
0,3	3,3 ± 0,18	3,5 ± 0,23	3,5 ± 0,21	3,5 ± 0,15
0,4	3,4 ± 0,18	3,4 ± 0,15	3,3 ± 0,12	3,5 ± 0,15
0,5	3,3 ± 0,29	3,1 ± 0,27	2,7 ± 0,15	3,3 ± 0,17

It should be noted that use of given component in an amount of 0.1 g/dm³ is reasonable, as it increases the titer of rhizobia the most. The same concentration of $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ is recommended for use at co-cultivation of *B. japonicum* M-8 with *A. brasilense* 410 since the increasing magnesium content in the medium from 0.1 g/dm³ to 0.4 g/dm³ the number of bacteria ad varied but remained within the error.

As a result of our studies the various concentrations of nutrients sources that ensure the maximum growth effect with minimum reasonable nutrients content in the medium for the co-cultivation of soybean rhizobia with azospirills were identified. Data obtained can be used as the basis for optimization of culture media for co-cultivation of *B. japonicum* and *A. brasilense*.