OPTIMIZATION OF CULTURAL MEDIA FOR JOINT CULTIVATION OF *BRADYRHIZOBIUM JAPONICUM* AND *AZOSPIRILLUM BRASILENSE*

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Perspective is the creation of microbial preparations based on mixed cultures diazotrophs particular which include *Bradyrhizobium japonicum* and *Azospirillum brasilense* bacteria [1; 2]. This is due to the fact that at joint cultivation of these nitrogen fixers their growth activity is increased, which promotes the obtaining of high titre of microorganisms in biological preparation. In addition, at pre-plant soybean inoculation by nodule bacteria together with azospirillums more nodules appear on the roots of plants, compared to monobacterization, and nitrogen fixation activity increases, which in turn contributes to obtaining additional yield of this legume.

An important and difficult stage of development of microbial preparations is the optimization of the environment for the cultivation of microorganisms, which are their active basis. Especially difficult this task is at simultaneous cultivation of two or more types of bacteria that have different needs for nutrition sources [3].

At this both the availability of specific components of the environment for each type of microorganisms and features of possible competition for certain compounds of nutrients should be ensured.

Based on the above, the goal of the study was the optimization of liquid culture medium content for joint growing of *B. japonicum* and *A. Brasilense* in periodic culture.

Materials and methods. Objects of the study: *B. japonicum* M-8 [4], *A. brasilense* 18-2 [5], *A. brasilense* 410 [6]. All the microorganisms were obtained from the National Collection of useful soil microorganisms of Institute of Agricultural Microbiology and Agroindustrial Manufacture of NAAS. We express our sincere gratitude to the authors for courtesy strains.

The number of *B. japonicum* was determined by plate method through depth seeding of suspensions in agar medium based on pea broth [7]. The number of *A. brasilense* was determined by plate method in agar

medium of such a composition, g/dm³: potato broth – 1, malic acid – 3.6, sucrose – 2.5; pH 7.2-7.4 (diluted by NaOH solution), agar-agar – 11 g.

Petri dishes were kept in an incubator at the temperature of (28.0±2.0)°C. Counting of *B. japonicum* colonies was performed on the tenth day, *A. brasilense* – on the fourth day.

The cultivation of bacteria was carried out in the vessels of 500 cm³ on the shaker at a frequency of 240 rpm. at the temperature of (28.0±2.0)°C. *B. japonicum* and *A. brasilense* seeding material were applied in the ratio 1: 1. The duration of cultivation is 72 hours.

The optimization of the medium was performed using an algorithm for determining the effects of changing factors on the process of joint cultivation of microorganisms *Bradyrhizobium* and *Azospirillum*. To do this, based on previously obtained data as for the impact of the sources of nutrients on the growth of *B. japonicum* and *A. Brasilense*, the optimization was performed using symmetrical plans all factors of which vary at the same number of levels. The optimization was performed using the method of orthogonal Latin rectangles [8-10]. The criterion of optimization was the number of viable bacterial cells.

Mathematical processing of experimental data was performed by calculating the influence effect on the number of viable cells for all levels of the studied factors. The effect of different levels influence was calculated by the difference of arithmetic average of process output (number of cells) in all versions, where the factor was at this level and the average value of each series of experiments by the formulas:

$$b_0 = \frac{\sum_{j=1}^N y_i}{N}, b_{ik} = \frac{\sum_{j=1}^N y_{ij}^k}{N/m} - b_0$$

where b_0 - arithmetic average of the number of bacteria in all variants;
 $\sum_{j=1}^N y_i$ - total number of bacteria in all variants of the experiment;

b_{ik} - effect of each level;
 y_i – output in the i -th variant of planning;
 N – total number of variants in the plan;
 m – number of levels of each factor;
 $\sum_{j=1}^N y_{ij}^k$ - the amount of outputs in those variants of the plan, where i -th factor was on the k -th level.

Planning matrix was constituted according to the scheme $3 \times 3 \times 3$ (Table 1), which allowed to study mutual influence of three factors on the growth of *B. japonicum* and *A. brasilense* on three levels in the conditions of one experiment. Medium after the first phase of optimization was chosen for the basis, which includes: molasses, fodder yeast (dry), glucose, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, CaCO_3 .

Table 1. Scheme of experiment planning with 3 factors and 3 levels of variation

Variant number j	Factors levels k		
	Factor i_1	Factor i_2	Factor i_3
1	1	1	1
2	2	1	2
3	3	1	3
4	1	2	2
5	2	2	3
6	3	2	1
7	1	3	3
8	2	3	1
9	3	3	2

Statistical analysis of experiment data was performed using the computer program Statistica 6.0.

Results and discussion.

The study of growth activity of *B. japonicum* and *J. brasilense* while cultivating in mixed culture was performed in the medium which included the sources of various nutrients, the impact of which we studied at the first phase of its optimization [11]. At the second stage the following levels of components of the medium were established:

- molasses: 10.0; 15.0; 20.0 g/dm³ (step – 5 g/dm³);

- fodder yeast: 3.0; 4.0; 5.0 g/dm³ (step – 1 g/dm³);
 - phosphates (1:1) ($\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$): 0.3; 0.4; 0.5 g/dm³ (step – 0.1 g/dm³).

Analyzing the obtained data of multifactor experiment (Table 2) it should be noted that the number of *B. japonicum* M-8 bacteria at the joint cultivation with *A. brasilense* 18-2 ranged from 1.68 to 6.61 billion cells/cm³. At this, the number of azospirillums was varying in a narrow range from 0.83 to 2.91 billion cells/cm³. While obtaining the inoculant for soybeans the focus should primarily be on the rhizobia titre, that's why optimal in this case, in our opinion, should be considered the medium, which is provided in version 6.

The effects of all factors influence on the number of *B. japonicum* M-8, which was cultivated together with *A. brasilense* 18-2 were deducted at their levels. It was found that the maximum effect for molasses in relation to the rhizobia was received at its concentration of 20 g/dm³ (table 3), in relation to azospirillums – at the concentration of 15 g/dm³ (table 4). Maximum effects as for the impact of fodder yeast in relation to both studied microorganisms were observed at the use of component concentration of 4.0 g/dm³. At the study of influence of different content of phosphate salts on the growth of rhizobia the greatest effect was obtained at the use of the minimum concentration of this component, and on the growth of *A. brasilense* 18-2 – at the use of the concentration of 0.5 g/dm³.

Table 3. Effects of multifactor experiment on optimization of nutrient medium for *B. japonicum* M-8

Medium components	Concentration, g/dm ³	Effect
Molasses	10.0	-0.82
	15.0	0.13
	20.0	0.69
Fodder yeast	3.0	-0.49
	4.0	1.16
	5.0	-0.67
$\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O} + \text{KH}_2\text{PO}_4$ (1:1)	0.3	1.22
	0.4	1.00
	0.5	-0.22

Table 2. The number of *B. japonicum* M-8 and *A. brasilense* 18-2 bacteria in the versions of multifactor experiment on optimization of nutrient medium, billion cells/cm³

Experiment variants	Medium components concentration, g/dm ³			Number of <i>B. japonicum</i> M-8 bacteria	Number of <i>A. brasilense</i> 18-2 bacteria
	Molasses	Fodder yeast	K ₂ HPO ₄ •3H ₂ O + KH ₂ PO ₄ (1:1)		
1	10	3	0.3	2.57	1.37
2	15	3	0.4	2.27	1.03
3	20	3	0.5	5.91	2.67
4	10	4	0.4	5.25	2.77
5	15	4	0.5	3.73	2.91
6	20	4	0.3	6.72	2.63
7	10	5	0.5	1.92	1.25
8	15	5	0.3	6.61	2.45
9	20	5	0.4	1.68	0.83

Table 4. Effects of multifactor experiment on optimization of nutrient medium for *A. brasilense* 18-2

Medium components	Concentration, g/dm ³	Effect
Molasses	10.0	-0.19
	15.0	0.14
	20.0	0.05
Fodder yeast	3.0	-0.30
	4.0	0.78
	5.0	-0.48
K ₂ HPO ₄ •3H ₂ O + KH ₂ PO ₄ (1:1)	0.3	0.16
	0.4	-0.45
	0.5	0.28

It should be noted that *B. japonicum* M-8 bacteria were more sensitive to changes in the concentrations of studied medium components in comparison with *A. brasilense* 18-2 at the cultivation of these microorganisms in a mixed culture.

The number of *B. japonicum* M-8 bacteria at the joint cultivation with *A. brasilense* 410 diazotrophs ranged from 0.76 to 3.20 billion cells/cm³. The number of azospirillums was varying in the range of 0.70 to 3.34 billion cells/cm³ at this. Optimum in this case to be considered the medium comprising of molasses at the concentration of 20 g/dm³, fodder yeast – 5 g/dm³, phosphates – 0.4 g/dm³ (table 5).

In the studies of the impact of all factors on the number of *B. japonicum* M-8 and *A. brasilense* 410 cultivated together, it was found that the maximum effect of molasses in relation to the studied microorganisms was observed at its concentration in the culture medium at the rate of 20 g/dm³, which represents its maximum studied value. In the study of the effects of other investigated components it was found (table 6-7) that the greatest impact on the number of grown bacteria was observed at maximum values of concentrations of these components - 5 g/dm³ of fodder yeast and 0.5 g/dm³ of the mixture of phosphate salts.

Table 6. Effects of multifactor experiment on optimization of nutrient medium for *B. japonicum* M-8, billion cells/cm³

Medium components	Concentration, g/dm ³	Effect
Molasses	10.0	-0.39
	15.0	0.18
	20.0	0.63
Fodder yeast	3.0	-0.16
	4.0	-0.11
	5.0	0.69
K ₂ HPO ₄ •3H ₂ O + KH ₂ PO ₄ (1:1)	0.3	-0.11
	0.4	0.01
	0.5	0.52

Table 5. The number of *B. japonicum* M-8 and *A. brasilense* 410 bacteria in the versions of multifactor experiment on optimization of nutrient medium, billion cells/cm³

Experiment variants	Medium components concentration, g/dm ³			Number of <i>B. japonicum</i> M-8 bacteria	Number of <i>A. brasilense</i> 410 bacteria
	Molasses	Fodder yeast	K ₂ HPO ₄ •3H ₂ O + KH ₂ PO ₄ (1:1)		
1	10	3	0.3	1.20	1.90
2	15	3	0.4	1.83	2.20
3	20	3	0.5	2.24	2.56
4	10	4	0.4	0.76	0.70
5	15	4	0.5	2.46	3.00
6	20	4	0.3	2.21	1.33
7	10	5	0.5	2.62	3.08
8	15	5	0.3	2.01	1.23
9	20	5	0.4	3.20	3.34

Table 7. Effects of multifactor experiment on optimization of nutrient medium for *A. brasilense* 410, billion cells/cm³

Medium components	Concentration, g/dm ³	Effect
Molasses	10.0	-0.11
	15.0	0.14
	20.0	0.41
Fodder yeast	3.0	0.22
	4.0	-0.32
	5.0	0.55
K ₂ HPO ₄ •3H ₂ O + KH ₂ PO ₄ (1:1)	0.3	-0.51
	0.4	0.08
	0.5	0.88

Based on these data, we can note that to achieve maximum impact on the growth of *B. japonicum* M-8 and *J. brasilense* 410 bacteria high concentrations of fodder yeast and phosphates are required.

Thus, by the results of multifactor experiment an optimized medium for joint growing of rhizobia and different strains of azospirillums was offered. It is shown that the effect of molasses, fodder yeast and phosphates influence on growth activity of *B. japonicum* depends not only on the amount of chemicals in the medium, but also on *A. Brasilense* strain which is jointly cultivated with.