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THE EFFECT OF CERIUM DIOXIDE NANOPARTICLES ON THE *Bradyrhizobium japonicum* POPULATION

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One of the effective approaches involves the use of biological compositions containing nitrogen-fixing bacteria, particularly from the genus *Bradyrhizobium*, and cerium dioxide nanoparticles (CDNs), which prove particularly promising for biological research due to their minimal toxicity. The object of the research was the soy rhizobia strain *Bradyrhizobium japonicum*, which was deposited in the D.K. Zabolotny Institute of Microbiology and Virology NAS of Ukraine under registration number IMV B–7194. The work used CDNs, particle size 4–6 nm, $\xi \approx +10$ mV. Methods of regression analysis of variance and experimental designs were used to assess dependence between bacterial titer of *B. japonicum* IMV B–7194 and several factors: CDNs concentration, substrate (mannitol) concentration and duration of culturing. Statistical analysis was carried out using TIBCO Statistica 14. On the basis of the obtained model, it was established that the duration of exposure (cultivation) of CDNs was the most important factor ($F = 8.08$, $p \leq 0.001$), with its increase, the number of cells increases linearly. At the same time, an increase in CDNs concentration ($F = 3.0722$, $p \leq 0.006$) should lead to a decrease in cell titer compared to the positive effect of mannitol ($F = 6.19$, $p \leq 0.001$), with other factors constant. It was determined that the effect of the studied nanoparticles in different concentrations had a non-linear dose-dependent nature.

Keywords: cerium dioxide nanoparticles, nitrogen-fixing bacteria, *Bradyrhizobium*.

INTRODUCTION

Today, among the pressing agriculture problems technogenic influence and overloading of soils, which lead to the death of their nitrogen-fixing biota and the appearance of extraneous, including pathogenic microorganisms, dangerous for plants and people, are attracting more and more attention. One of the consequences is also the imbalance of nitrogen and its transformations in agroecosystems. This process can have a negative impact on the development and yield of cultivated plants, in particular, the important leguminous fodder crop of soybeans (Suman *et al*., 2022).

Considering the need to grow soybeans and the problem of nitrogen imbalance in the soil, there is an urgent need to create modern ecological technologies. An effective method is the use of biological formulations (Hadas, 2014). Currently, the world market has a wide range of inoculants based on nitrogen-fixing bacteria for the processing of leguminous crops, including soybeans. Pre-sowing seeds inoculation with biological preparations based on nitrogen-fixers contributes too many factors, in particular: release of antibiotic substances by prokaryotes, which prevents infection by phytopathogens; increasing the photosynthesis activity; increasing the yield of the crop compared to the crop without treatment; increasing protein accumulation in legume seeds. In addition, the use of bacterial inoculants is an alternative to mineral nitrogen fertilizers, the production of which is quite energy consuming and expensive (Agbowuro *et al*., 2021).

Nodule bacteria are most often used for the production of inoculants. These microorganisms have an individual selective ability to infect different types of leguminous

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plants, i.e. specificity to a certain species. Thus, only bacteria of the genus *Bradyrhizobium* are able to form symbiotic relationships with soybeans and nodules on the roots of the plant (Bogino *et al*., 2015). Therefore, the basis of inoculants is often the species *Bradyrhizobium japonicum*, which is actively used in the production of bacterial inoculants for the pre-sowing treatment of soybean seeds. In addition, this species is used by science as a model organism for which genetic engineering methods are actively being implemented to create new highly efficient nitrogen-fixing strains capable of increasing the yield of leguminous crops (Sundh *et al*., 2021). However, the slow growth rate of *B. japonicum* presents several challenges in agricultural biotechnology, particularly in the fields of microbial inoculants (Krutylo, 2016).

In recent years, the use of nanoparticles in agriculture is gaining more and more popularity (Pansambal *et al*., 2023). Nanoparticles are defined as particles smaller than 100 nm in size. In particular, nanoparticles, taking part in electron transfer processes, enhance the action of enzymes that convert nitrates into ammonium nitrogen, intensify cell respiration, photosynthesis, synthesis of enzymes and amino acids, carbohydrate and nitrogen metabolism (Nosrati *et al*., 2023).

It should be noted that nanocrystalline materials with a wide spectrum of action include nanobiomaterials based on cerium dioxide. Despite considerable interest, the biological activity of cerium dioxide has not been sufficiently studied. Until recently, almost no attention was paid to this compound, since cerium dioxide is insoluble in water and biological fluids. Due to oxygen non-stoichiometry and low toxicity, cerium dioxide nanoparticles (CDNs) are extremely promising objects for biological research (Younis *et al*., 2016; Zhang *et al*., 2019).

The aim of our study was to research a possible mathematical dependence between microbial growth of *Bradyrhizobium japonicum*, concentration CDNs, bioprocess time and mannitol concentration.

MATERIALS AND METHODS

Bradyrhizobium japonicum strain IMB B-7194 from the collection of the D.K. Zabolotny Institute of Microbiology and Virology NAS of Ukraine. An aqueous colloidal solution of CDNs (particle size 4-6 nm, $\xi \approx +10$ mV) was obtained by hydrothermal treatment of the solution for 50 hours in the temperature range of 100÷200°C.

Cultivation of *B. japonicum* was carried out in Erlenmeyer flasks with a volume of 750 ml (with a medium volume of 100 ml) with periodic cultivation at 220 rpm at a temperature of 28-30˚C, for 72-96 hours in a mannitol-yeast liquid nutrient medium (g/l): NaCl – 0.1; $K_2HPO_4 \cdot 3H_2O$ – 0.5; $MgSO_4 \cdot 7H_2O$ – 0.2; $FeCl_3$ – 0.01; mannitol – 10.0; calcium gluconate - 1.5; yeast extract – 2.0; pH 7.0. At the initial stages of cultivation, the initial optical density of the suspension of microorganisms was $OD_{670} = 0.1$.

All studies were carried out in at least three repetitions. The obtained data were statistically processed by generally accepted methods of variation statistics.

The dependence of the growth of *B. japonicum* bacteria on the participation of CDNs was evaluated using regression analysis methods in the theory of experimental planning according to the full factorial plan 3^k. This approach makes it possible to estimate the linear and quadratic effects of factors (X_1, X_2, X_3) on the indicator Y within the framework of one model and to express it in the form of a regression equation:

$$y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{i=1}^{n} \sum_{j>i}^{n} a_{ij} x_i x_j \quad (1)$$

where: a_0 – constant, a_i – linear coefficient, a_{ii} – quadratic coefficient, and a_{ij} – second-order interaction coefficient.

Statistical processing of the data of the planned experiment (calculation of regression coefficients, analysis of variance (ANOVA) and construction of response surfaces was carried out using the trial version of the Statistica program (TIBCO Software Inc., https://www.tibco.com/) using the DOE library. Coefficients were considered statistically significant at $p \leq 0.05$.

RESULTS

During the cultivation of *B. japonicum* in a liquid nutrient medium with different amounts of CDNs, the dynamics of cell titer growth of the studied bacteria were established. Taking into account the data of previous studies, we used nanoparticles in the most physiologically expressed concentrations of 1 μM and 1 mM for biotesting the effect of CDNs. In the course of the work, *in silico* optimization of the parameters of the mathematical model of the response of *B. japonicum* to the presence of CDNs was carried out. The study was conducted according to the plan of a full factorial design (FFD) (Table 1).

Table 1. Mathematical model factors for assessing the CDNs impact

Factors	Factor levels		
	-1	0	+1
Concentration of cerium dioxide nanoparticles (X_1), M	0	10^{-6}	10^{-3}
Duration of action of cerium dioxide nanoparticles (X_2), days	3	10	17
Concentration of mannitol in nutrient medium (X_3), g/l	5	10	15

The plan of the experiment to study changes in the intensity of the number of *B. japonicum* bacteria at different concentrations of CDNs, as well as options for combining factors and the obtained results are shown in Table 2.

Table 2. Experiment planning matrix according to the FFD

№	Optimization factors			Optimization parameter
	X_1 *	X_2 *	X_3 *	Cell titer, CFU/ml
1	0	3	5	1,60E+07
2	0	3	10	2,40E+08
3	0	3	15	2,14E+08
4	0	10	5	4,40E+08
5	0	10	10	9,00E+08
6	0	10	15	2,20E+09
7	0	17	5	1,40E+09
8	0	17	10	1,80E+09
9	0	17	15	3,50E+09
10	0,000001	3	5	1,83E+07
11	0,000001	3	10	1,04E+08
12	0,000001	3	15	5,60E+08
13	0,000001	10	5	3,80E+08
14	0,000001	10	10	1,25E+09
15	0,000001	10	15	1,40E+09
16	0,000001	17	5	7,40E+08
17	0,000001	17	10	2,54E+09
18	0,000001	17	15	4,90E+09
19	0,001	3	5	1,50E+07

№	Optimization factors			Optimization parameter
	X_1 *	X_2 *	X_3 *	Cell titer, CFU/ml
20	0,001	3	10	8,90E+07
21	0,001	3	15	1,30E+08
22	0,001	10	5	2,90E+08
23	0,001	10	10	6,20E+08
24	0,001	10	15	1,26E+09
25	0,001	17	5	4,30E+08
26	0,001	17	10	7,53E+08
27	0,001	17	15	1,90E+09

* Note:
X_1 – concentration of cerium dioxide nanoparticles, M
X_2 – duration of action of cerium dioxide nanoparticles, days
X_3 – concentration of mannitol in the nutrient medium, g/l

The level of significance of the effects (linear, quadratic and interaction effects) was determined by the analysis of variance (ANOVA), which showed that the concentration of cerium dioxide nanoparticles (X_1) and the duration of action of nanoparticles (X_2) had a significant ($p \leq 0.02$) effect on the cell titer, but lower than the concentration of mannitol ($p \leq 0.01$). For a visual assessment of the effects of variance analysis, a Pareto diagram is presented in (Fig. 1), on which the effects are arranged in descending absolute value. This diagram shows that the linear effects of the duration of cultivation and mannitol concentration have the maximum reliable effect, and the effect of CDNs has the minimum.

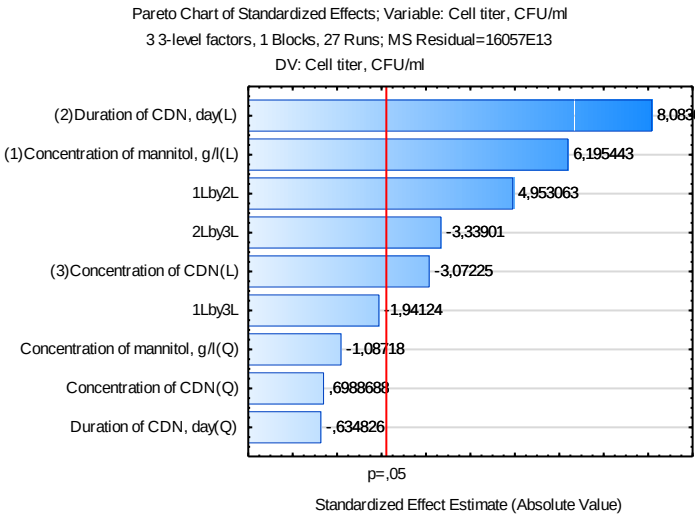

Figure 1. The influence of the researched factors on the *B. japonicum* titer

During the initial data analysis, a regression equation was obtained, which has the form of a quadratic polynomial of the second order, taking into account only statistically significant effects:

$$Y = 9{,}43\times10^8 - 5{,}8\times10^8 X_1 + 1{,}62\times10^9 X_2 + 1{,}24\times10^9 X_3 - 6{,}63\times10^8 X_1\times X_2 - 1{,}14\times10^9 X_2\times X_3 \quad (2)$$

From the obtained model, it turns out that the duration of CDNs exposure (cultivation) turned out to be the most important factor (F = 8.08, $p \leq 0.001$), with its increase, the number of cells should increase linearly. At the same time, an increase in CDNs concentration (F = 3.0722, $p \leq 0.006$) should lead to a decrease in cell titer compared to the positive effect of mannitol (F = 6.19, $p \leq 0.001$), with other factors constant.

According to the response surface of the titer of *B. japonicum* bacteria (Fig. 2), the effect of low CDNs concentrations only increases with increasing duration of cultivation with nanoparticles. The obtained data allow us to assert the positive effect of micromolar concentrations of CDNs on the growth of bacteria, but less pronounced than the effect of mannitol.

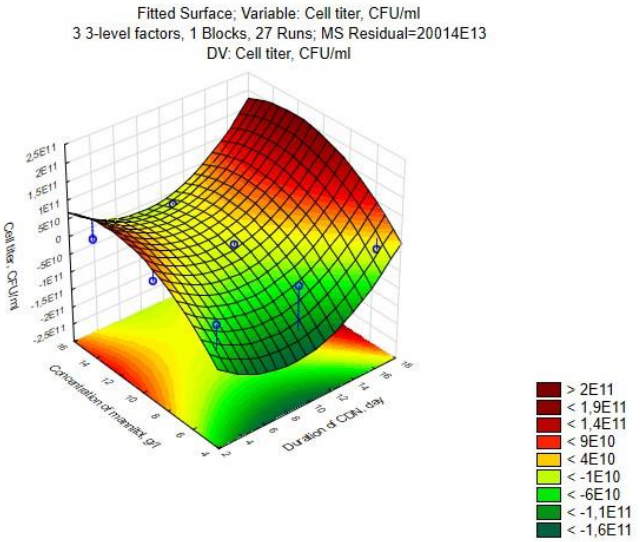

Figure 2. The response surface of *B. japonicum* titer, as functions of the studied factors

The conducted work made it possible to determine the optimal values for the studied factors within the framework of this study. (Fig. 3).

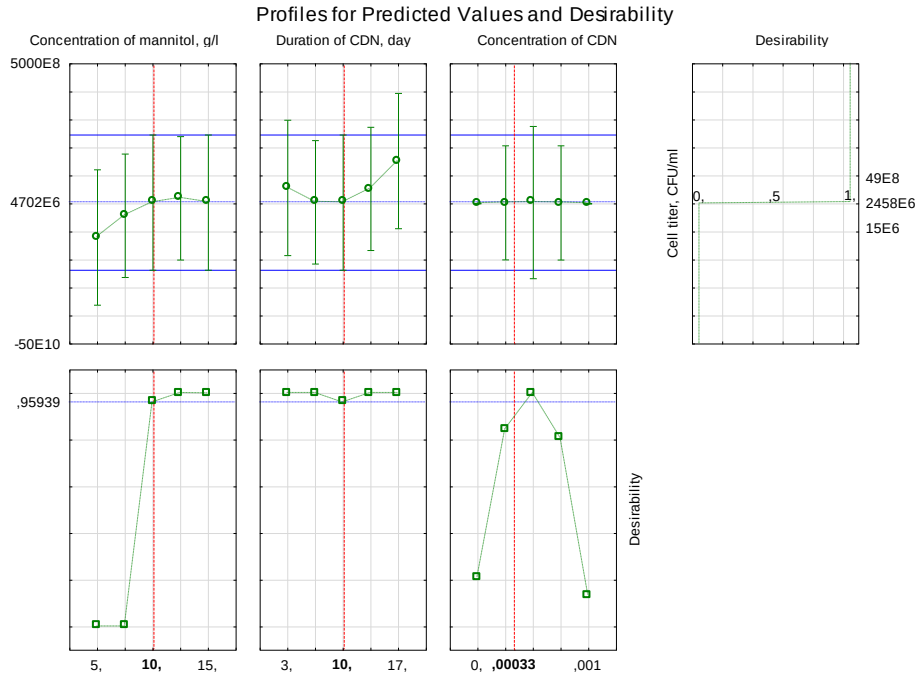

Figure 3. Profiles of predicted values to obtain optimal values of the studied factors

As a result of our work, the stimulating effect of low concentrations of CDNs on the growth of the studied bacteria was shown, which is enhanced by the presence of a substrate in the medium. The calculated maximum cell titer at these levels of exposure was determined at

a nanoparticle concentration of 330 μmol and a mannitol concentration of 10 g/L in the medium during a ten-day exposure to the CDNs.

CONCLUSIONS

Throughout the course of this study, results were obtained that enabled the formulation of several conclusions:

Based on bacterial growth indicators, it was determined that cerium dioxide nanoparticles at concentrations ranging from 1 micromole to 1 millimole had the most significant effect on cell titer after their introduction into the culture medium, as compared to the control group ($p < 0.01$).

- The absence of a direct concentration dependence between CDNs and the ability to grow was noted.

- As part of our experimental work, we obtained optimal value calculations for the investigated factors, which revealed: the duration of CDNs action is 10 days, with a concentration of nanoparticles of 330 μmol and a concentration of mannitol in the medium of 10 g/l.

Thus, the use of the model gram-negative microorganism *B. japonicum* allows for biotesting of cerium dioxide nanoparticles, and makes it possible to assess their effect in the concentration range of 1 micromole to 1 millimole. The research is promising and requires comprehensive study for the creation of new nanotechnologies and the possible application of these nanomaterials in complexes with microorganisms during the inoculation of nodular nitrogen-fixing bacteria in the pre-sowing processing of soybeans.

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