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Optimization of *Azotobacter chroococcum* IMV B-7836 cultivation and evaluation of its effectiveness as a bioinoculant of cucumber

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Abstract: An important stage in the development of an effective biological preparation for agricultural crops is the selection of an optimal nutrient medium that would meet the physiological needs of microorganisms and ensure the implementation of their agronomically important functions as biological agents of the inoculant. The growth characteristics of *A. chroococcum* IMV B-7836 in liquid media of different chemical composition were studied. The optimal digest medium for the production of the preparation was selected, the chemical compositions of which provides the maximum titer of *A. chroococcum* IMV B-7836 in the optimal terms of diazotroph cultivation. We studied the dependence of the generation of the number of viable cells of *A. chroococcum* IMV B-7836 on the time of cultivation in batch culture on modified molasses medium, Ashby's, and pea medium. It was shown that bacterization of cucumber seeds with a new effective inoculant based on *A. chroococcum* IMV B-7836 promotes better growth and development of plants, and increases nitrogenase activity in the root zone of vegetable plants and an increase in yield during early and presowing bacterization. Upon early and presowing bacterization of cucumber seeds, nitrogen-fixing activity was at the level of 46.1–47.6 nmol C_2H_4/g dry soil per hour in the flowering phase. The yield of the Konkurent variety cucumber upon early and presowing treatment with an inoculant based on *A. chroococcum* IMV B-7836 for three years increased by 15.1% compared to the control.

Keywords: *Azotobacter chroococcum,* cultivation, digest medium, early bacterization, presowing bacterization, nitrogen-fixing activity, yield.

Introduction

Molecular nitrogen fixation is an important biological process that, along with photosynthesis, ensures the existence of life on the planet (Kots, 2021; Ladha et al., 2022). The main role in the process of nitrogen fixation is played by diazotrophs, whose relationships with plants are quite diverse and are the subject of study of modern microbiology, genetics, biochemistry, and plant physiology (Mesquita et al., 2023).

The research conducted in recent decades around the world and, in particular, in Ukraine has shown that soil nitrogen-fixing bacteria are capable of providing tens of millions of tons of biological nitrogen to the soil annually (Antonella Di Benedetto et al., 2017; Mahmud et al., 2020). The research of the properties of diazotrophs, their ability to form symbioses and associations with plants, and ways to optimize the introduction of various types of beneficial microorganisms into the root zone of agricultural crops remains relevant today (Cordeiro and Echer, 2019; Grzyb et al., 2021). At the same time, one of the most difficult issues is the regulation of the growth and functional activity of diazotrophs (Kozar, 2021).

Bacteria of the genus *Azotobacter* are known to form associations with plants, as they actively develop in the root zone of various plants (Inomura et al., 2016). Another advantage of *Azotobacter* is their ability to maintain viability in the form of cysts for several years under the influence of low temperature, drought, and other adverse environmental influences (Hindersah et al., 2020; Aasfar et al., 2021). These bacteria can positively influence plant growth and development due to their ability to fix molecular nitrogen from the atmosphere (Inomura et al., 2018). However, the nitrogenase activity of individual strains of the genus *Azotobacter* can vary significantly (Zhengtao et al., 2019).

The efficiency of inoculation largely depends on the ability of microorganisms to maintain viability and high physiological activity under the influence of environmental factors (Kozieł, 2023).
Therefore, the creation of an effective biological preparation for agricultural crops involves the selection of microorganisms with a complex of beneficial properties that are able to withstand negative environmental influences, as well as the selection of an optimal digest medium for microorganisms that will satisfy their physiological needs and reveal the agronomically valuable properties of microorganisms–biological agents of the inoculant.
Therefore, the purpose of this study was to study the growth characteristics of the new strain *A. chroococcum* IMV B-7836 (Bilokonska and Kozar, 2020) in synthetic and semi-synthetic media, in particular in an in-house modified industrial medium to obtain a high titer of nitrogen-fixing bacteria, as well as to study nitrogenase activity and study the efficiency of *A. chroococcum* IMV B-7836 for inoculation of vegetable crops.

Results

Assessing the peculiarities of growth of *A. chroococcum* IMV B-7836 on synthetic and semi-synthetic media

The primary objective of the study was to assess the peculiarities of growth of *A. chroococcum* IMV B-7836 on synthetic and semi-synthetic media, as well as in an industrial medium, which we modified to obtain a high titer of nitrogen-fixing bacteria in the inoculum. The composition of the medium used for the cultivation of bacteria of the genus *Azotobacter* in industry was taken as the base for the latter, except for corn extract, due to its high content of nitrogenous substances, which is an unfavorable factor for the activity of diazotrophs (Bahulikar et al., 2020; Imran et al., 2021).
In order to meet the needs of diazotrophs in organic substances, the amount of molasses in the medium was increased by 2 times compared to the industrial one. Since a relatively high content of sugars in the medium is known to induce intensive production of bacterial biomass (Oros et al., 2011). The concentration and composition of salts also have a significant effect on the growth of microorganisms (Roi et al., 2016; Bissenova et al., 2024). Considering the nutritional needs of bacteria of the genus *Azotobacter*, K_2HPO_4 (0.025%); KH_2PO_4 (0.025%); $MgSO_4 \cdot 7H_2O$ (0.02%); $CaCO_3$ (0.3%); NaCl (0.02%) were added to modified molasses medium (Roi et al., 2016).
We studied the dependence of the generation of the number of viable cells of *A. chroococcum* IMV B-7836 on the time of cultivation in batch culture on modified molasses medium (No. 1), Ashby's (No. 2) and pea medium (No. 3).
It should be noted that the lag phase of *A. chroococcum* IMV B-7836 in the medium with molasses was the shortest — 48 hours, which is due to the better adaptation of bacteria to the growing conditions. On the medium No. 2 and No. 3, the lag phase lasted 60 hours. It is probable that the carbon compounds that make up the molasses contribute to the growth of associative bacteria, reducing the duration of bacterial adaptation. After the lag phase, an exponential phase was registered. During this period, diazotrophs are particularly sensitive to environmental conditions (Bertrand, 2019), which affect the maximum titer and the duration of the specified phase. On the in-house modified medium (Figure 1), we obtained the highest titer – 9.3 billion cells after 72 hours of cultivation. On the synthetic Ashby's medium, the maximum cell titer was also registered after 72 hours of cultivation, but it was 2.2 times lower compared to the titer obtained during cultivation on the medium No. 1, namely 4.2 billion (Figure 2). On the semi-synthetic medium with pea broth, the titer of the studied strain also reached the maximum level 72 hours after cultivation, but was only 0.5 billion cells (Figure 3).
The exponential phase when grown on the medium No. 1 and No. 2 occurred after 48 hours of cultivation, and when grown on the medium No. 3 — at 60 hours of cultivation.
In the period from 24 to 72 hours, the curves characterizing growth in the three media had a close slope, although the number of cells increased more intensively in the medium with molasses. These data indicate that in the medium with molasses at 72 hours, the number of *Azotobacter* cells showed no further increase, i.e., the exponential growth phase ended and the stationary phase began.
It is probable that the shorter time before the exponential phase (i.e., faster adaptation of bacteria), as well as the highest titer of the bacterial suspension in the in-house modified medium, are due to the high concentration of polysaccharides in molasses (Chu and Barnes, 2016; Bertrand, 2019).
Therefore, we have selected the optimal digest medium for the industrial production of biomass, which will further be used as the main component of the preparation, whose chemical composition provides the maximum titer of *A. chroococcum* IMV B-7836 in the optimal period of cultivation of diazotrophs. Thus, the maximum number of CFU of *A. chroococcum* IMV B-7836 was 9.3 billion at 72 hours of cultivation.

Efficiency of presowing bacterization of cucumber seeds with *A. chroococcum* IMV B-7836

The cultivation of crops using safe technologies involves the use of microbial preparations, the bioagents of which are microorganisms resistant to adverse environmental influences (Ríos-Ruiz et al., 2020; Aasfar et al., 2021). We have studied the ability of diazotrophs to maintain viability on the surface of cucumber seeds for 30 days and provide a positive effect on plant productivity. The seeds were inoculated 30 days (early) and 2 hours (presowing bacterization) before sowing into the soil. The effect of inoculation on nitrogenase activity in the root zone of plants was studied. It was shown that the difference in nitrogen-fixing activity in the rhizosphere of plants during the studied developmental phases in the variant with early and presowing treatment varied within the measurement accuracy, which indicates the high viability of *A. chroococcum* IMV B-7836 (Figure 4). The highest rate of nitrogenase activity was reported upon the presowing bacterization of cucumber seeds with the suspension of *A. chroococcum* IMV B-7836 in the flowering phase, which was 47.6 nmol C_2H_4/g dry soil per hour.

Discussion

The regulation of the growth of microorganisms is known to be possible by selecting optimal cultivation media, which allows for a significant increase in biomass (Bissenova et al., 2024). The selection of the composition of the medium is crucial during the development of bacterial inoculants and should ensure the production of a sufficient number of viable and active microbial cells (Lobo et al., 2019; Bashan et al., 2013).

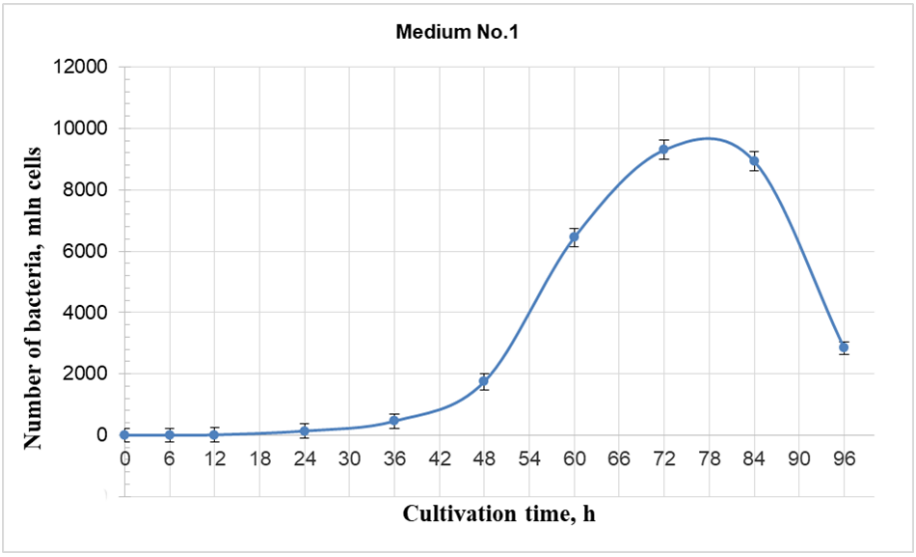

Figure 1. Growth of *A. chroococcum* IMV B-7836 on the modified molasses medium

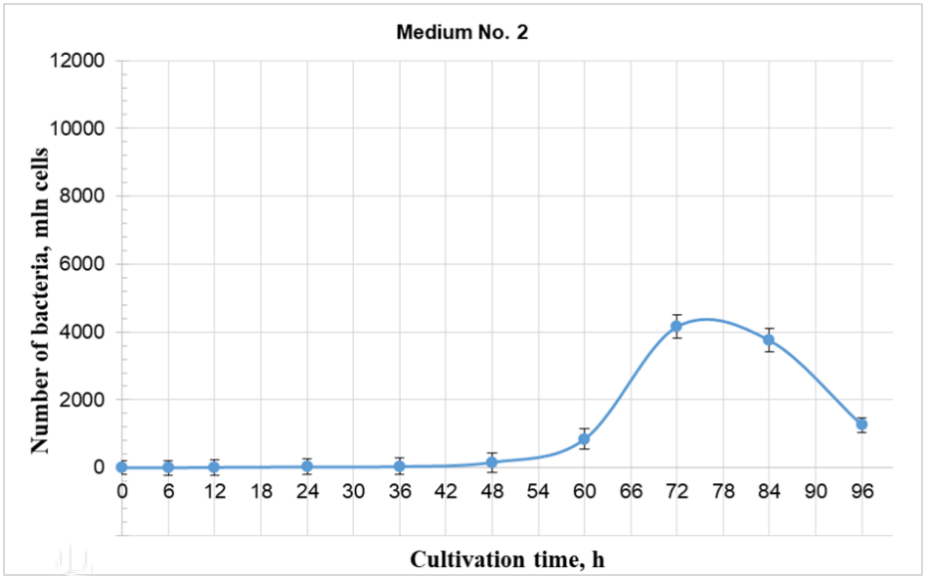

Figure 2. Growth of *A. chroococcum* IMV B-7836 on the Ashby's medium

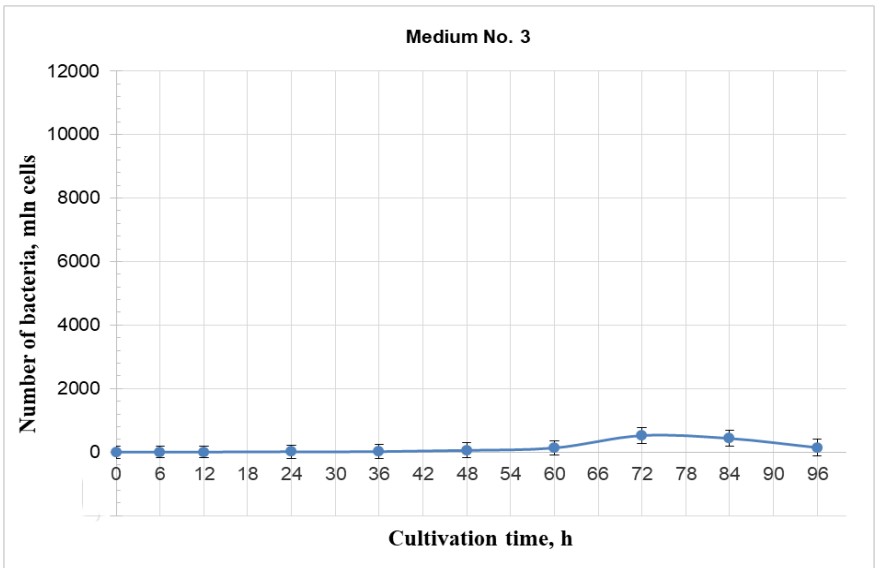

Figure 3. Growth of *A. chroococcum* IMV B-7836 on the pea medium

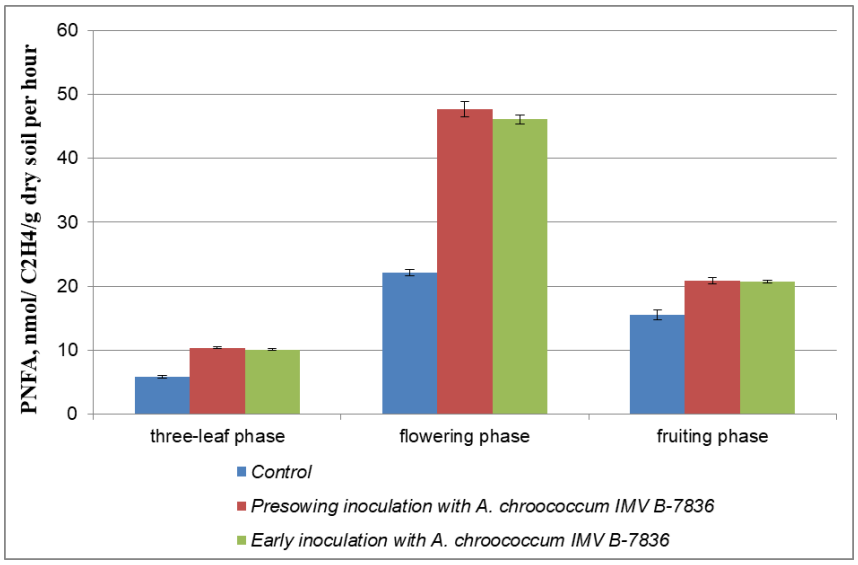

Figure 4. The effect of bacterization on the potential nitrogen-fixing activity of rhizosphere soil in the cucumber plants of Konkurent variety, *field experiment*

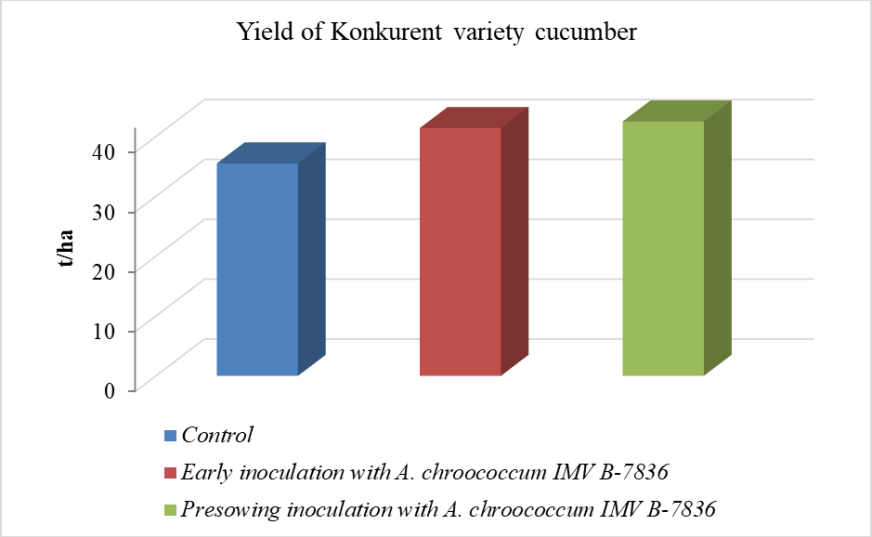

Figure 5. The effect of bacterization on the yield of Konkurent variety cucumber*,*field experiment*
**LSD (0.05) 1.0 = significant differences against control at the level of 0.05*

The study of the effect of bacterization on the yield of the Konkurent cucumber variety after early and presowing treatment with an inoculant based on *A. chroococcum* IMV B-7836 for three years showed that this strain is active and viable, as it contributes to an increase in yield by 15.1% versus the control (Figure 5).

Synthetic and semi-synthetic media of various compositions containing glucose as a carbon source and inorganic salts as a source of trace elements (Mo^{2+}, Mn^{2+}, Fe^{2+}, and Ca^{2+}) are used for the cultivation of *Azotobacter* (Morgun et al., 2011; Oros et al., 2011; Roi et al., 2016). Oros et al. (2011), in their studies, showed that the number of *A. chroococcum* cells on Ashby's synthetic medium reaches a maximum concentration of $6.4 \bullet 10^8$ mL. Morgun et al. (2011), in the patent "Method of growing spring wheat using *Azotobacter chroococcum* T79", noted that when grown on this medium, the bacterial titer reached 10^8 cells per 1 mL at 72 h of cultivation.

According to the results of our studies, it was shown that the cultivation of *A. chroococcum* IMV B-7836 on Ashby's medium (No. 2) provided a higher number of CFU ($4.2 \bullet 10^9$ mL) at 72 h of cultivation. Liquid nitrogen-free synthetic Ashby's medium is not advisable to use for obtaining biomass of bacteria of the genus *Azotobacter* due to the slow growth rate of bacteria.

Literature sources have evidence that semi-synthetic digest media with the addition of pea decoction (Kozar et al., 2009) or molasses (Oros et al., 2011; Roi et al., 2016) are also used for industrial production of *Azotobacter* biomass. When using such media, researchers obtained cell titers from $7.6 \bullet 10^8$ to $1.07 \bullet 10^{10}$ cells/mL at 24–72 hours of cultivation.

We have shown that upon the industrial cultivation of *A. chroococcum* IMV B-7836, it is optimal to use a medium with molasses, because the bacterial titer was $9.3 \bullet 10^9$ cells at 72 hours of cultivation, while on a semi-synthetic medium with pea decoction, it was only $5 \bullet 10^8$ cells.

For the effective use of inoculants with a high initial bacterial titer in microbial preparations for presowing treatment of vegetable seeds, an equally important indicator is the activity and viability of the microbial agent (Bahulikar et al., 2021; Aasfar et al., 2021; Haskett et al., 2021), and especially their ability to survive on seeds in case of early treatment (Kozar, 2021). As a result of our studies, it was shown

that *A. chroococcum* IMV B-7836 is active viable because, in the case of early treatment, the nitrogen-fixing activity of the strain remains at the level of presowing treatment: 46.1 and 47.6 nmol C_2H_4/g dry soil per hour in the flowering phase.
Genus *Azotobacter* is commonly used for presowing inoculation of agricultural crop seeds to increase crop yield (Inomura et al., 2016; Hindersah et al., 2020; Ríos-Ruiz et al., 2020; Aasfar et al., 2021; Kozieł, 2023). Researchers from different countries of the world used Azotobacter-based biofertilizers for presowing bacterization of cucumber seeds and obtained an increase in its yield from 5 to 30% (Sabier et al., 2015; Nagaraja Kusagur et al., 2022), depending on the type of soil and growing technology.
Over three years of research, the yield of Konkurent variety cucumbers upon early and presowing treatment with the inoculant based on *A. chroococcum* IMV B-7836 increased by 15.1% versus the control.

Materials and methods

Laboratory experiments
Purity of the cultures and their viability was assessed according to generally accepted methods of microbiological research (Abdel-Hamid et al., 2010; Philippot et al., 2012; Wakarera et al., 2022).
To select the composition of the digest medium that would provide the growth needs and biological activity of the strain, the growth characteristics of the highly effective *A. chroococcum* IMV B-7836 were studied on three different media of synthetic and semi-synthetic composition.
For the cultivation of *A. chroococcum* IMV B-7836, the following media were used:

- Medium No. 1 (g/L): molasses – 60.0; K_2HPO_4 – 0.25; KH_2PO_4 – 0.25; $MgSO_4 \cdot 7H_2O$ – 0.2; $CaCO_3$ – 3.0; NaCl – 0.2; pH 7.0–7.2;
- Medium No. 2 (g/L): sucrose – 20; $K_2HPO_4 \cdot 3H_2O$ – 0.2; $MgSO_4 \cdot 7H_2O$ – 0.2; NaCl – 0.2; K_2SO_4 – 0.1; CaCO3 – 3.0; pH 7.0–7.2;
- Medium No. 3 (g/L): pea decoction – 1000; sucrose – 10.0; $(NH_4)_2SO_4$ – 1.0; K_2HPO_4 – 0.5; KH_2PO_4 – 0.5; $MgSO_4 \cdot 7H_2O$ – 0.2; $CaCO_3$ – 2.0; pH 6.8–7.0.

Cultivation was carried out on rockers (240 rpm) at 28°C for 96 hours. At intervals of 6–12 hours, the number of viable cells was measured by serial dilutions and plating of *A. chroococcum* IMV B-7836 on Ashby's agar medium (No. 2) with subsequent counting of colony-forming units (CFU).

Field experiments

A field experiment was conducted at the Institute of Agricultural Microbiology and Agro-industrial Manufacture of National Academy of Agricultural Sciences of Ukraine in Chernihiv (northern region of Ukraine).
The potential nitrogenase activity of rhizosphere soil and washed roots was determined by the acetylene method (Haskett et al., 2021) on a gas chromatograph HP 4890A.
Seeds of cucumber variety Konkurent were treated with 13.4 mL of working mixture per 1.0 kg of cucumber seeds at the rate of 35,000 cells per seed. In the control variant, the seeds were treated with tap water.
The placement of plots in the field experiment was randomized. The repetition of variants was fourfold. The area of the experimental plot was 10 m^2. The area of the accounting plot was 7.2 m^2. The crop was sown in early May according to the 80x20 cm scheme. The agricultural technology of growing cucumbers was carried out according to generally accepted requirements for the Polissia zone of Ukraine.
Meadow-chernozem leached light loam soil, which contains from 2.8% to 3.4% humus (according to Tyurin), from 0.27% to 0.31% total nitrogen, about 15 mg/100 g of soil P_2O_5 (according to Kirsanov), from 13 mg/100 g of soil to 16 mg/100 g of K_2O (according to Maslova) and, pH water 6.2. Variants with optimal soil moisture (60% PVG) and variants with insufficient water supply (30% PVG) are provided.
The climate of the Chernihiv region is moderately warm and mild with sufficient humidity. The average annual air temperature is +5.7 … +6.6. The average air temperature of the coldest month (January) in the region is -6.2 ... -7.6, and the warmest (July) fluctuates within +18.4 ... +19.7.
The Chernihiv region receives an average of 550-650 mm of precipitation per year. The highest monthly rainfall occurs in June–July, the lowest in January–March. The amount of precipitation in some years ranges from 400 to 850 mm. The highest daily rainfall sometimes reaches 100-140 mm.
The scheme of the field experiment to study the effect of bacterization of cucumber seeds with *A. chroococcum* IMV B-7836 on the growth and development of plants was as follows:
1. Control.
2. Early bacterization of *A. chroococcum* IMV B-7836 (30 days before sowing).
3. Presowing bacterization with a new *A. chroococcum* IMV B-7836 (2 hours before sowing).
Experimental data processing was carried out using mathematical statistics methods.

Conclusion

It was found that the maximum number of CFU of *A. chroococcum* IMV B-7836 was 9.3 billion (at 72 hours of cultivation) when cultivated on the medium with molasses. As a result of three-year field studies, it was found that early and presowing bacterization of cucumber seeds with *A. chroococcum* IMV B-7836 resulted in the level of nitrogen-fixing activity of 46.1–47.6 nmol C_2H_4/g dry soil per hour in the flowering phase. The yield of Konkurent variety cucumber upon early and presowing treatment with the inoculant based on *A. chroococcum* IMV B-7836 bacteria increased by 15.1% versus the control.

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