VIABILITY OF *Azotobacter chroococcum* IMB B-7836 AND THEIR INFLUENCE ON CUCUMBER PRODUCTIVITY

Oksana BILOKONSKA[*], Serhii KOZAR[*]

[*] *Institute of Agricultural Microbiology and Agroindustrial Manufacture of the NAAS, Laboratory of Microbial Physiology, Chernihiv, Ukraine. Correspondence author: Oksana Bilokonska, Institute of Agricultural Microbiology and Agroindustrial Manufacture of the NAAS, Laboratory of Microbial Physiology 97, Shevchenka Str., zip code:14027, Chernihiv,Ukraine, phone: +380462231749, fax: +380462232157, e-mail: obilokonska@ukr.net*

Abstract. One of the tasks of modern biotechnology is the development of highly efficient inoculants based on beneficial soil bacteria that are capable of producing high stable yields. The bioagents of such inoculants may be soil diazotrophs, which play an important role in agrocenoses by fixing atmospheric nitrogen and transforming the latter into a plant-accessible form. The objective of our work was to study the viability and functional activity of *Azotobacter chroococcum* IMB B-7836 and to assess its effect on cucumber plants, namely on the nitrogen-fixing activity in the root zone of the plant and yield. It was found that three months after treatment, the number of bacterial cells on the seeds stored at 4 °C was significantly higher than in the variant with the storage of seeds at 28 °C. A significant negative effect of UV exposure on the viability of *A. chroococcum* IMB B-7836 was established. To reduce the negative effects of UV exposure during bacterization, cells of azotobacter in the form of cysts were used, which ensured the prolongation of the viability of microbial cells on the seeds until sowing in the soil. It was found that nitrogen-fixing activity in the root zone of cultivaitng cucumbers is higher with *A. chroococcum* IMB B-7836 in the form of cysts as compared with vegetative cells of *Azobacter*. Bacterization contributed to the increased productivity of cucumber plants that is more likely associated with improved nitrogen nutrition of this culture; at the same time, the highest cucumber yield was reported for pre-sowing treatment of seeds with *Azobacter* in the form of cysts. These findings may be applied to improve efficacy of seed bacterization affected by unfavourable environmental factors, in particular temperature and UV.

Keywords: *Azotobacter chroococcum*; cysts; temperature; UV exposure; viability.

INTRODUCTION

Today, there is an increased interest in the use of biopreparations based on soil nitrogen fixing bacteria as an alternative to chemical fertilizers in agricultural manufacture. These microorganisms contribute to increased crop yields by fixing atmospheric nitrogen, improving plant nutrition and quality of crop products, without polluting the environment [1, 3]. One of the representatives of diazotrophs is *Azotobacter*, which actively fixes atmospheric nitrogen and converts it into a plant-accessible form [10].

Introduction of *Azotobacter* into the rhizosphere of plants occurs by treatment of seeds with inoculum, but under the influence of environmental factors (in particular, adverse temperatures, UV exposure) bacteria can die in the period from bacterization to the beginning of crop development. Decrease in the number of *Azotobacter* cells on seeds can adversely affect the efficacy of bacterization [13].

UV exposure is part of the natural solar spectrum, which has a positive effect on plant seeds, providing stimulation of seed germination. When seeds are exposed to UV irradiation, the permeability of biological membranes of cells changes resulting in the stimulation of the initial growth processes [18], however the problem is the inactivation of cells of bacterial inoculant applied directly to the seeds [20].

Efficient introduction of beneficial soil microorganisms into agrocenosis by inoculation of seeds is important for modern agriculture. For this purpose, the effect of abiotic factors on the inoculum should be investigated, since the functional activity of the bacteria depends on environmental conditions, in particular temperature and light [9].

MATERIAL AND METHODS

The studies were conducted with *Azotobacter chroococcum* IMB B-7836. This strain is deposited in the Depository of the Institute of Microbiology and Virology of the NAAS of Ukraine.

Ashby medium of the following composition (g/dm^3) was used for cultivation and maintenance of the culture [34]: glucose – 20.0 g/dm^3; $KH_2PO_4 \times 2H_2O$ – 0.2 g/dm^3; KH_2PO_4 – 0.2 g/dm^3; $MgSO_4 \times 7H_2O$ – 0.2 g/dm^3; NaCl – 0.2 g/dm^3; K_2SO_4 – 0.1 g/dm^3; $CaCO_3$ – 5.0 g/dm^3; tap water up to 1.0 dm^3, pH 6.8 to 7.0.

To obtain vegetative cells, the bacteria were cultivated in Ashby liquid culture medium. The test microorganisms were grown for 72 hours under conditions of periodic cultivation on a microbial shaker at 220 rpm at 28 ºC.

To obtain cysts, *A. chroococcum* IMB B-7836 was cultured on the surface of agar Ashby medium in Petri dishes. Petrie dished were placed in a thermostat at 28 °C. After 7 days, the dishes were exposed to a temperature of 44 °C [6, 19].

Staining of cysts of *A. chroococcum* IMB B-7836 was carried out according to the method proposed by O. Wyss [37]: 8.5 cm^3 of acetic acid, 3.3 cm^3 of disodium sulfate (Na_2SO_4), 200 cm^3 of neutral red, 50 cm^3 of light green and 50 cm^3 of ethyl alcohol were sequentially added to a 100 cm^3 of sterile water. The solution was stirred constantly, then filtered through a paper filter. Bacteria were suspended in the dye and subjected to microscopy [29, 37].

Experimental design No. 1. Temperature exposure of *A. chroococcum* IMB B-7836 included four parts:

I. Storage of seeds at 4 ± 2 ºC.

II. Storage of seeds at 12 ± 2 ºC.

III. Storage of seeds at 20 ± 2 ºC.

IV. Storage of seeds at 28 ± 2 ºC.

The following variants are provided in each part of experiment:

1. Seed bacterization with vegetative cells of *A. chroococcum* IMB B-7836.
2. Seed bacterization with cells of *A. chroococcum* IMB B-7836 in the form of cysts.

Cucumis sativus (cucumber variety - Konkurent) seeds were bacterized with *A. chroococcum* IMB B-7836. Baseline bacterial count was 3.5×10^4 cells per seed. Bacterized seeds were stored in a place protected from light at 4 ± 2 ºC; 12 ± 2 ºC; 20 ± 2 ºC; 28 ± 2 ºC.

Bacterial count on seeds was measured at Day 30, 60 and 90 by sequential dilutions method followed by cultivation on agarized Ashby medium [26, 32].

Experimental design No. 2. UV exposure of *A. chroococcum* IMB B-7836 on cucumber seeds included eight parts:

I. Irradiation of *A. chroococcum* IMB B-7836 cells during 5 sec.

II. Irradiation of *A. chroococcum* IMB B-7836 cells during 10 sec.

III. Irradiation of *A. chroococcum* IMB B-7836 cells during 30 sec.

IV. Irradiation of *A. chroococcum* IMB B-7836 cells during 60 sec.

V. Irradiation of *A. chroococcum* IMB B-7836 cells during 300 sec.

VI. Irradiation of *A. chroococcum* IMB B-7836 cells during 600 sec.

VII. Irradiation of *A. chroococcum* IMB B-7836 cells during 1,200 sec.

VIII. Irradiation of *A. chroococcum* IMB B-7836 cells during 1,800 sec.

The following variants are provided in each part of experiment:

1. Seed bacterization with vegetative cells of *A. chroococcum* IMB B-7836.
2. Seed bacterization with cells of *A. chroococcum* IMB B-7836 in the form of cysts.

Distance from irradiation source was 100 cm.

The bacterial count after irradiation on seeds was determined by sequential dilution method followed by cultivation on agarized Ashby medium [26, 32].

Field experiments were conducted at the study site of Institute of Agricultural Microbiology and Agroindustrial Manufacture of the NAAS.

Variants of field experiment:

1. Control.
2. Pre-sowing bacterization with *A. chroococcum* IMB B-7836 (vegetative cells).
3. Early bacterization with *A. chroococcum* IMB B-7836 (vegetative cells).
4. Pre-sowing bacterization with *A. chroococcum* IMB B-7836 (cysts).
5. Early bacterization with *A. chroococcum* IMB B-7836 (cysts).

An inoculant was made on the basis of *A. chroococcum* IMB B-7836 to perform the field experiment. Bacterization of agricultural seeds by microbial inoculant was carried out before sowing and in advance three months before sowing

Sod weakly podzolic soil, containing 1.2 % of humus (by Tiuryn), 5 mg to 6 mg/100 g soil of mobile nitrogen P_2O_5 (by Tiuryn and Kononov), 11-12 mg/100 g of soil P_2O_5 (by Chirikov), 12 mg/100 g soil to 13 mg/100 g soil K_2O (by Maslova) [17, 21]. The area of the accounting plot is 10.4 m^2, the repetition is four-fold.

The efficacy of bacterization was tested in field experiments with Konkurent variety of cucumber plants. Planning and conducting of field experiments, crop accounting and statistical processing of the obtained data were performed according to existing procedures [5, 16, 31].

Determination of the activity of nitrogen fixation of microorganisms was performed by acetylene method [8, 33, 35] on HP 4890A Hewlett Packard gas chromatograph.

Data were subjected to factorial analyses of variance (ANOVA) with Excel. Then, the differences between the means were compared by Fisher's Least Significant Difference (LSD) test at a probability level of 95%. Significance levels were expressed as $p = 0.05$ and data were significant when $p < 0.05$.

RESULTS

Microbial life depends on temperature, due to the fact that all organisms are made of chemical components and all life processes occur on the basis of chemical reactions subject to the laws of thermodynamics [28].

The results of the studies show a better maintenance of the number of *A. chroococcum* IMB B-7836 on seeds under prolonged exposure to lower temperatures. For example, Figure 1 shows that after three months, azotobacter had the best viability at 4 °C, the number of viable cells in the form of cysts was 3.7 thousand cells/seed, and vegetative cells - 0.5 thousand cells/seed.

It was found that the number of *Azotobacter* cells in the form of cysts under storage of seeds at a temperature of 4 °C at Day 90 was 3 times lower than at Day 30. The number of cells at 28 °C decreased sharply after storage for 30 days and amounted to 2.4 thousand cells/seed, and at Day 90 upon storage of the inoculated seed at 28 °C, no viable vegetative cells were found. The number of cells in the form of cysts was 185 cells/seed at Day 90 at a temperature of 28 °C (Fig. 1).

An important environmental factor that adversely affects the bacterization of seeds is exposure to UV irradiation. The analysis of the data obtained indicates that *Azotobacter* cysts had higher viability than vegetative cells.

The study found that *A. chroococcum* IMB B-7836 remained viable on the seeds under exposure to UV radiation for 60 sec in all variants of the experiment.

Considering study findings over a period from 5 sec to 60 sec, we have prolonged the irradiation time of bacterial seeds to 1800 sec, and further experiment was performed in the following time algorithm: 300 sec, 600 sec, 1200 sec, 1800 sec (Fig. 2).

Azotobacter had the best seed viability on seeds in the variant with cells in the form of cysts. For example, the results of bacterial survival in this variant indicate that within 300 sec the cell count decreased from 35 thousand cells/seed to 14.4 thousand cells/seed, or by 41.1 %, and continued to decrease with increasing time of UV exposure.

The effect of bacterization on the nitrogen-fixing activity of cucumber was established. It was found that the highest nitrogen-fixing activity was during bacterization with *A. chroococcum* IMB B-7836 cysts before sowing of cucumber seeds: it was 59.7 nmol of C_2H_4/g of dry soil/hour in flowering phase, which exceeds control by 95.1 %. In the variant with pre-sowing bacterization using *A. chroococcum* IMB B-7836 vegetative cells, this parameter exceeded the control by 61.1 % (Table 1).

It was found that the lowest yield of 35.5 t/ha was in the control. The yield of 41.1 t/ha was provided by the early bacterization using *A. chroococcum* IMB B-7836 vegetative cells. In the variant with pre-sowing bacterization using *A. chroococcum* IMB B-7836 cells in the form of cysts, the yield was 48.0 t/ha (Table 2)

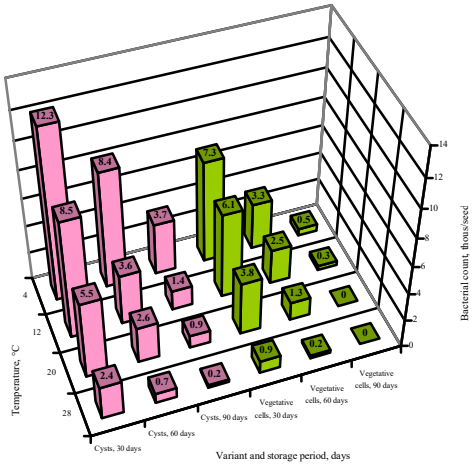

Figure 1. Maintenance of *A. chroococcum* IMB B-7836 on seeds exposed to a temperature range from 4 ± 2°C to 28 ± 2°C

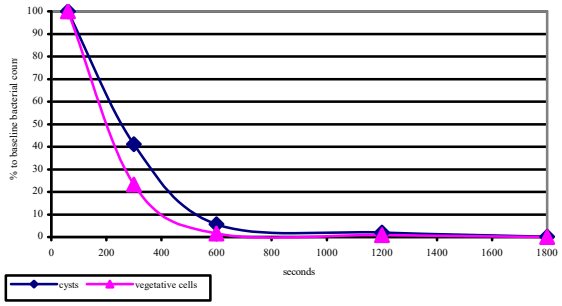

Figure 2. Influence of UV exposure to viability of *A. chroococcum* IMB B-7836 on cucumber seeds.

Table 1. Influence of bacterization on nitrogen-fixing activity of cucumber variety Konkurent in flowering phase, field experiment

Variants of experiment	PNFA (potential nitrogen-fixing activity), nmol C_2H_4/g of dry soil/hour	Increase	
		nmol C_2H_4/g of dry soil/hour	%
Control (without treatment)	30.6	-	-
Pre-sowing bacterization with *A. chroococcum* IMB B-7836 (vegetative cells)	49.3*	18.7	61.1
Early bacterization with *A. chroococcum* IMB B-7836 (vegetative cells)	44.3*	13.7	44.8
Pre-sowing bacterization with *A. chroococcum* IMB B-7836 (cysts)	59.7*	29.1	95.1
Early bacterization with *A. chroococcum* IMB B-7836 (cysts)	52.8*	22.2	72.5
LSD $_{(0.05)}$	1.3		

* = significant differences at the level of 0.05

Table 2 Influence of bacterization on yield of cucumber variety Konkurent, field experiment

Variants of experiment	Yield, t/ha	Increase	
		t/ha	%
Control	35.5	-	-
Pre-sowing bacterization with *A. chroococcum* IMB B-7836 (vegetative cells)	42.5*	7.0	19.7
Early bacterization with *A. chroococcum* IMB B-7836 (vegetative cells)	41.4*	5.9	16.6
Pre-sowing bacterization with *A. chroococcum* IMB B-7836 (cysts)	48.0*	12.5	35.2
Early bacterization with *A. chroococcum* IMB B-7836 (cysts)	43.0*	7.5	21.4
LSD $_{(0.05)}$	1.4		

* = significant differences against control at the level of 0.05

DISCUSSION

Azotobacter bacteria, including *A. chroococcum*, are able to fix atmospheric nitrogen, ensuring its entry to agrocenoses. This is confirmed both by our studies and works of other researchers [4, 12, 14]. When nitrogen-fixing bacteria are introduced to agrocenoses, their activity and viability is affected by various environmental factors, in particular radiation and temperature [11, 23, 36]. As a result of our studies, we have found that exposure to UV irradiation has a negative effect on the survival of *A. chroococcum* IMB B-7836. Cells in the form of cysts maintain their viability better than vegetative cells. Furthermore, the results obtained are consistent with those of Shemshura et al. (2019) [27], the researchers have found that exposure to different doses of UV irradiation caused photoreactivation of *Azotobacter* cells. At the same time, we discovered for the first time that viable cells of azotobacter were present on cucumber seeds even after 1200 sec of UV irradiation. The number of vegetative cells on the seeds was lower compared to the number of cysts. Berleman & Bauer (2004) [2] have described that cysts are more resistant to radiation, temperature, and UV irradiation as opposed to vegetative cells. To improve the survival of *A. chroococcum* IMB B-7836, it is advisable to use bacterial cell cysts that have a positive effect on maintenance of azotobacter cells, when exposed to environmental factors (temperature and UV irradiation).

Therefore, a negative effect of temperature > 12°C on the viability of *A. chroococcum* IMB B-7836 on seeds was detected. The negative effect of UV exposure on the azotobacter directly applied to the seeds was established. To increase the efficacy of the inoculant, cells of *A. chroococcum* IMB B-7836 in the form of cysts were used.

Better survival rate of *Azotobacter* cysts compared with vegetative cells under the exposure to various factors may be explained by the fact that cyst has thickened multilayer cover which protects bacterial cell from unfavourable environmental factors [7].

A. chroococcum IMB B-7836 strain is able to fix atmospheric nitrogen. Under pre-sowing bacterization with the latter, nitrogen-fixing activity in rhizospheric soil was 59.7 nmol of C_2H_4/g of dry soil/hour. Crop yield depends on nitrogen-fixing potential of the bacteria. For example, Kuts has described in his work the use of microbial preparations based on nitrogen-fixing *Azotobacter* bacteria that ensure increased yield of marketable root crops of red beet by 16–23 % [15]. Pantsireva et al. provides the yield of green bean obtained in the variants of experiment, where *Azotobacter*-containing biopreparation was used. For example, the yield was 29.1 t/ha, and it was higher than the control variant by 13.0 t/ha [22]. Although other data are available in terms of positive action of *Azotobacter* on vegetable cultures and atmospheric nitrogen fixation in the soil [24, 25, 30], however, we have studied the influence of representative of these bacteria, namely *A. chroococcum* IMB B-7836 strain in the form of cysts and vegetative cells, on the cucumber. Cucumber yield in the variant with pre-sowing bacterization using *A. chroococcum* IMB B-7836 cysts was the highest. Increased yield may be explained by the fact that *Azotobacter* in the form of cysts has better survival rate, thus it fixes nitrogen more intensely. This probably improves nitrogen nutrition of plants. The data obtained can be used to ensure the survival of *Azotobacter* cells on seeds under exposure to adverse environmental factors, in particular temperature and UV irradiation, and to increase the efficacy of inoculants applied directly to the seeds.

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