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ABILITY OF *LACTOBACILLUS PLANTARUM* ONU 12 AND *BACILLUS MEGATERIUM* ONU 484 TO STIMULATE GROWTH OF WHEAT SEEDLINGS AND TO FORM BIOFILMS

Development of biological preparations for organic agriculture should include the study of interactions of microorganisms – the components of biopreparations, with representatives of natural microbiota of agrocoenoses. As a microorganism – typical representative of epiphytic and soil microbiota, species Bacillus megaterium has been selected. ***Aim.*** *The study of effect of Lactobacillus plantarum ONU 12 and Bacillus megaterium ONU 484 on germination and growth of wheat seedlings.* ***Materials and Methods.*** *Seeds of wheat Triticum aestivum L. were inoculated with suspensions of bacteria B. megaterium ОНУ 484, L. plantarum ОНУ 12 and their mixture was germinated in hydroponics and soil under green house conditions. Germination of seeds, mean length of roots and height of seedlings were compared. Capability of these microorganisms to form biofilms was studied: inoculated seedlings were dyed with 0.1% acridin orange and observed with a magnification x600.* ***Results.*** *Simultaneous inoculation with bacteria L. plantarum ONU 12 and with the representatives of soil microbiota B. megaterium ONU 484 didn't decrease the stimulation effect of lactobacilli. Opposite, the highest stimulation effect on plants both in hydroponics and in soil caused the treatments with the mixture L. plantarum ONU 12 + B. megaterium ONU 484 and with the strain B. megaterium ONU 484 alone. In hydroponics mean length of roots increased in 8.0 – 16.9%, mean height of seedlings – in 8.8 – 24.3%. In soil germination of seeds increased in 7.0%, mean height of seedlings – in 7.6%, mean root length – in 13.1%. Lactobacilli and bacilli in the mixture were able to form biofilms with a developed matrix.* ***Conclusions.*** *In presence of representative of soil microbiota B. megaterium ONU 484 phytostimulation the activity of lactobacilli increased. Strain B. megaterium ONU484 also showed stimulation activity when applied separately.*

Key words: *stimulation of plant growth, Lactobacillus plantarum, Bacillus megaterium.*

The development of biological preparations for organic agriculture should include the study of interactions of microorganisms from biological preparations with representatives of natural microbiota of agrocenoses. Lactobacilli isolated from plants are known for their stimulatory activity on plant growth [9; 10], which is explained by the synthesis of auxin hormones or their precursors described in

the literature [6]. It remains unclear whether the stimulatory effect of lactobacilli decreases when bacteria penetrate into the natural environment and interact with representatives of plant and soil microbiota. As a microorganism – a typical representative of the epiphytic and soil microbiota there were selected the species *Bacillus megaterium* in our study. The representatives of the genus *Bacillus* inhabit the surface and vessels of plants, as well as soil, and are known for their ability to stimulate plant growth [6; 8; 10; 13]. The phytostimulative activity of bacilli is explained by the possible synthesis of auxins, cytokinins, gibberellins and their precursors [8; 12; 13]. Gruneberg et al. (2006) indicate the successful application of the consortium of *Bacillus subtilis* and *Lactobacillus spp.* to improve the growth of ornamental plants. The previous studies described strains – representatives of the genera *Bacillus* and *Lactobacillus* with stimulatory activity for each strain separately [3; 9].

The aim of this work was to study the effect of *Lactobacillus plantarum* and *Bacillus megaterium* on germination and growth of wheat seedlings.

Materials and methods

Strains from the Collection of the Department of Microbiology, Virology and Biotechnology of Odesa National I. I. Mechnikov University *L. plantarum* ONU 12 і *B. megaterium* ONU 484 were used. Lactobacilli were grown in a liquid MRS medium [5] at 37 °C overnight and used in the experiments with a culture concentration of 10^8 cells/ml. Bacilli were grown overnight in LB medium [4] at 28 °C. To prepare the mixture, bacteria of both strains were grown to the concentration of 10^8 CFU/ml, and mixed in a ratio of 1:1.

For simulating the conditions of hydroponics, we used the Aquasave S gel, prepared according to the instructions. As a test plant, *Triticum aestivum* L. (wheat variety – Kuialnik) was used. Seed surfaces were sterilized by 25% hydrogen peroxide for one minute. Then seeds were washed three times in sterile distilled water.

Overnight cultures of lactobacilli and bacilli at concentration of 10^8 cells/ml were used to prepare dilutions 1% (10^7 cells/ml), 0.1% (10^6 cells/ml), 0.01% (10^5 cells/ml), 0.001% (10^4 cells/ml), 0.0001% (10^3 cells/ml), 0.00001% (10^2 cells/ ml) for each strain and for mixtures of strains. Seeds were soaked for one hour in each of the prepared dilutions. Subsequently, seeds were transferred in Aquasave S gel in glassware for germination. The control was seeds soaked for one hour in sterile distilled water. Germination was carried out in a greenhouse in Aquasave S gel for seven days.

With the concentrations of bacteria that exhibited the higher stimulatory activity we conducted the same experiment in soil conditions (Fig. 1). We used the soil "Poliskiy Universalniy" with a high content of peat, which was not sterilized before sowing.

In total in three independent experiments there were tested 300 seeds of each variants. After seven days, the growth characteristics of plants were measured: average lengths of the roots of seedlings and average height of the plant [2].

Statistical processing was performed using Excel package. The lengths of the roots and shoots as quantitative values were expressed as a mean and confidential

interval at 95%, the germination as a qualitative value was expressed as a percentage and a standard error. Significant differences between control measurements and inoculated seedlings were detected in Student's t-test ($P < 0.05$).

To study the ability of bacteria to form biofilms obtained wheat seedlings were washed from gel in distilled water. Biofilms were fixed in 96% ethanol for 15 min and stained with 0.1% solution of acridine orange for 10 minutes. The colored seedlings were dried on a slide and examined under a microscope. For observation, the microscope PrimoStar PC, Carl Zeiss with a total magnification x600 was used. The level of formation of biofilm was evaluated by the scale given in Table 1 [1].

Table 1

The evaluation criteria of the formation of biofilms [1]

Criteria	Description
–	Does not form biofilms
+	Individual attached cells without the formation of biofilms
+ +	Individual well-formed microcolonies
+ + +	Well-formed biofilms with gaps in the structure
+ + + +	Well-formed biofilm with matrix

We investigated 10 samples of each variant and evaluated the level of biofilm formation in 10 fields of vision. The biofilms were photographed using the camera Canon EOS 500D.

Results and Discussion

Inoculation of wheat seeds with bacteria of the strains *L. plantarum* ONU 12 and *B. megaterium* ONU 484 influenced germination and growth of seedlings in hydroponics conditions. Thus, treatment with bacilli at concentrations from 0.1% (10^6 cells/ml) to 0.001% (10^4 cells/ml) of the overnight cultures significantly increased the germination (Table 2).

Table 2

Germination of wheat in gel (%) after the treatments with *L. plantarum* ONU 12 and *B. megaterium* ONU 484 in different concentrations, cells/ml

Variant of treatment	Concentration, cells/ml				
	10^7	10^6	10^5	10^4	10^3
L. plantarum ONU 12	65.0 ± 1.2	84.2±1.0*	72,4±1,3	67.1 ± 2,8	66.5 ± 1.2
B. megaterium ONU 484	40.0±3.6	63.3±3.6*	90,0±2,2**	73.3±3.3***	55.0±2.6
L. plantarum 12 + *B. megaterium* 484	53.3±3.7	60.0±3.6	57.0±3.6	57.0±3.6	55.0±2.6
Water (control)	55.0±2.6%				

Note: the difference between the values is reliable at P = 0.05; * – compared with the corresponding indicator at concentration of 10^7 cells/ml; ** – at concentration of 10^6 cells/ ml; *** – at concentration of 10^5 cells/ml; all values are significantly different from control.

In the control – seeds, soaked in water instead of the suspension of bacteria, the germination was 55.0 ± 2.6%. We can see that low concentrations of bacteria (10^3 cells/ml) did not influence the germination. A high concentration (10^7 cells/ml) did not influence or even suppress germination, as in the case with *B. megaterium* ONU 484. Application of the individual strains was more effective than treatment with the mixture of strains *L. plantarum* ONU 12 and *B. megaterium* ONU 484 (Table 1). Treatment with lactobacilli improved the germination in 5.0–25.0%, bacilli – in 8.3–35.0% (except for the concentration of 10^7 cells/ml), and the mixture – in 1.7–5.0% (almost did not influence).

Measuring of the average length of the roots of seedlings showed that treatment of wheat seeds by suspensions of *B. megaterium* 484 increased root length in 8.0–14.8% (Fig. 1, A). In this case, the best concentrations were 0.1% (10^6 cells/ml) and 0.0001% (10^3 cells/ml) of *B. megaterium* 484 overnight cultures.

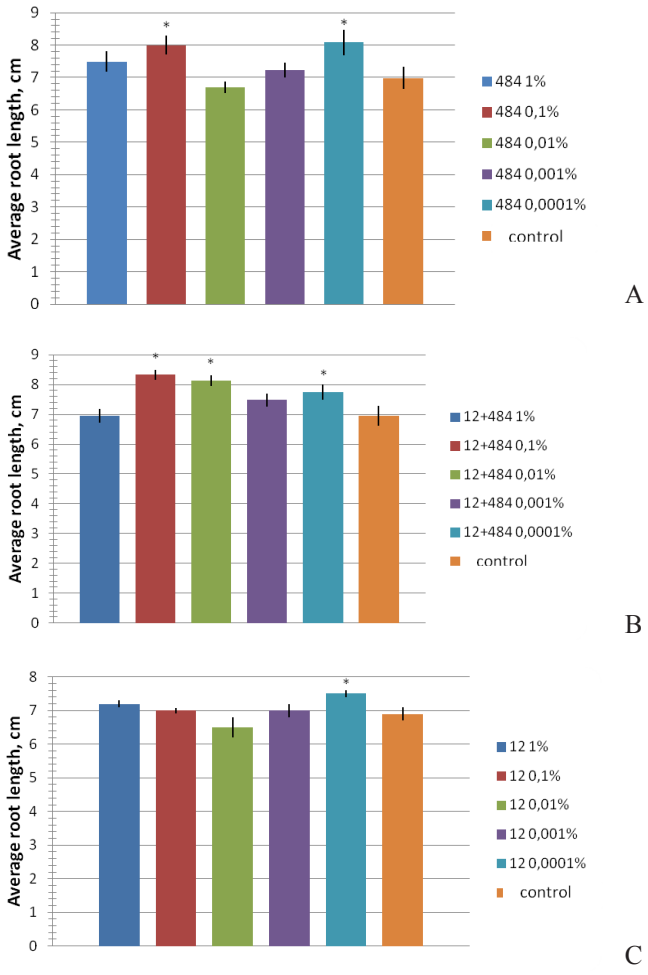


Fig. 1. Average length of the roots of wheat seedlings treated with bacterial suspensions and germinated in gel:
A – *B. megaterium* ONU 484; B – *L. plantarum* ONU 12;
C – *L. plantarum* ONU 12 + *B. megaterium* ONU 484,
* values are significantly different from the control (P <0.05)

Treatment with lactobacilli increased the length of roots in 8.0% (Fig. 3, B). The average length of roots of seedlings after treatment with the mixture of lactobacilli and bacilli in gel increased in 8.0–17.0% (concentration of applied bacterial suspensions 10^4–10^6 cells/ml) (Fig. 5). This means, that interaction with representatives of soil and plant microbiota – bacilli, did not decrease the stimulatory activity of lactobacilli. Opposite – their activity increased due to stimulatory effect of bacilli.

Figure 2 shows the wheat seedlings in gels after the treatment by the mixture of lactobacilli and bacilli.

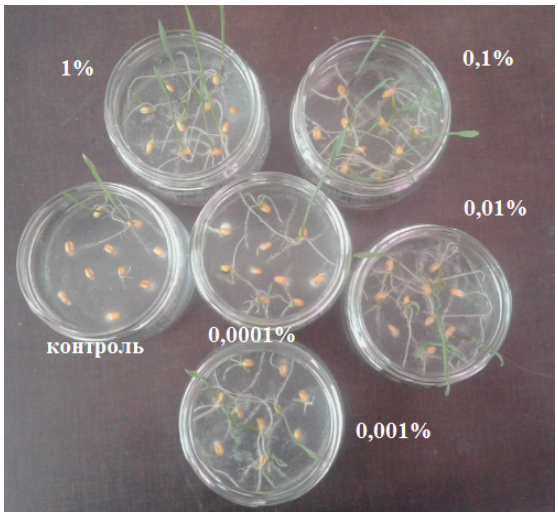


Fig. 2. Growth of root systems of wheat seedlings after the treatment with the mixture *L. plantarum* ONU 12 and *B. megaterium* ONU 484

Bacterial suspensions caused more significant effect on the height of seedlings. Thus, treatment with strain *B. megaterium* 484 increased the average height of seedlings in 8.8–21.4% (for concentrations of applied bacterial suspensions 10^3–10^6 cells/ml) (Fig. 3, A).

Treatment with lactobacilli increased the height of wheat seedlings in 10.4–12.7% (10^3–10^4 cells/ml) (Fig. 3, B). Treatments with the mixture also proved to be effective, increasing the height of seedlings in 8.8–24.3%, (10^3–10^7 cells/ml). When seeds were germinated in gel, treatment with the mixture of lactobacilli and bacilli was more effective than treatment with a separate strain of lactobacilli. Treatment by the strain *B. megaterium* ONU 484 also led to significant stimulation of plant growth. All concentrations caused positive effect, but application of the mixture was more effective at higher concentrations of bacteria (10^7–10^5 cells/ml), and use of bacteria of the strain *B. megaterium* ONU 484 individually could occur both at high concentrations (10^7 cells/ml) and at less concentrations – from 0.01% – to 0.0001% concentrations of daily culture (from 10^2 to 10^4 cells/ml).

The phenomenon of enhancement of the properties of lactobacilli under the influence of another strain is known for the synthesis of bacteriocins, when the so-called strain-inducer intensified the synthesis of antagonistic compounds, and

the strain-inducer did not necessarily belong to the species *L. plantarum,* and not necessarily – to the genus *Lactobacillus.* This could be *Escherichia coli, Bacillus spp.* and others [11]. Perhaps this phenomenon is also known for the synthesis of plant growth hormones by lactobacilli. The enhancement of the stimulating activity of lactobacilli in the mixture is still not described in the literature, and therefore its mechanisms require further study.

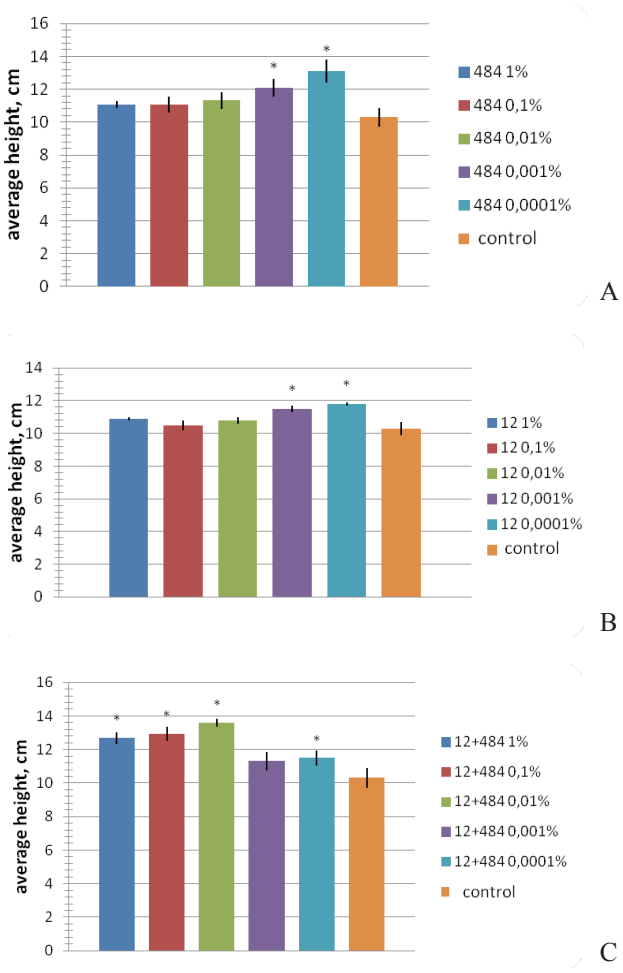


Fig. 3. Average height of wheat seedlings treated with bacterial suspensions and germinated in gel:
A – *B. megaterium* 484, B – *L. plantarum* ONU 12;
C – *L. plantarum* 12 + *B. megaterium* 484,
* values are significantly different from the control (P <0.05)

We have carried out the experiments on germination of inoculated seeds in the soil with the concentrations of bacterial suspensions that had the best stimulating effect in gel. As it is shown in Table 3, the effect of inoculation with bacteria was not so significant as in gel.

Only bacteria *B. megaterium* 484 at concentration of 0.01% (10^5 cells/ml) increased germination in 7% (in the control group germination reached 83.0%).

Table 3

Germination of wheat seeds in soil after the treatments with strains *L. plantarum* ONU 12 and *B. megaterium* ONU 484 at different concentrations, %

Strain	Concentration, cells/ml	Germination
B. megaterium ONU 484	10^7	70.0±3.7
	10^5	90.0±2.4*
	10^3	88.0±2.6**
L. plantarum 12 + *B. megaterium* 484	10^6	83.0±3.1
	10^5	85.0±2.9

Note: * Differences in comparison with the corresponding indicators, obtained at: * – concentration of 1%; ** – concentration 0.001% (P = 0.05); all values are significantly different from the control.

Growth indices at some concentrations in soil were even less than in the control, that indicates that these concentrations were too high for application in soil (Fig. 4), in contrast to the growth conditions in gel.

Thus, the average length of roots of wheat seedlings increased in soil after the treatment with 1% (10^7 cells/ml) *B. megaterium* 484 and a mixture of *L. plantarum* 12 + *B. megaterium* 484 at concentration of 0.01% (10^5 cells/ml) – an increase in 6.3 – 13.1% occured (Fig. 4).

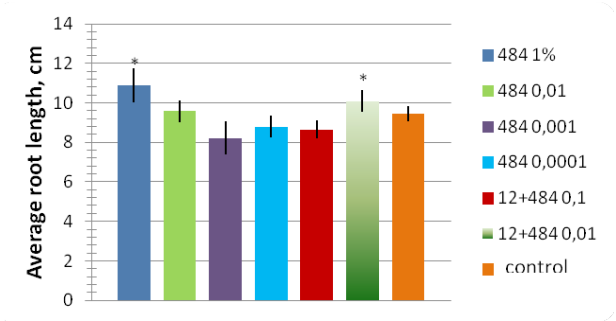


Fig. 4. Average length of roots of wheat seedlings, inoculated by bacteria and germinated in soil:
* values are significantly different from the control (P <0.05)

The most positive effect on average height of seedlings was caused by the suspension of 0.0001% (10^3 cells/ml) of the overnight culture of *B. megaterium* 484 – an increase of 7.6% occured (Fig. 5).

So, for germination in soil more careful selection of bacterial concentrations for seed treatment was required. The length of roots and the height of the plants were better influenced by treatment with an individual strain *B. megaterium* 484, moreover, different concentrations caused a different effect on the growth of the roots and the aboveground parts. For increasing the length of the root higher concentrations of bacteria (10^6 cells/ml) are required, for increasing the height lower concentrations – 0.0001% (10^3 cells/ml) could be proposed.

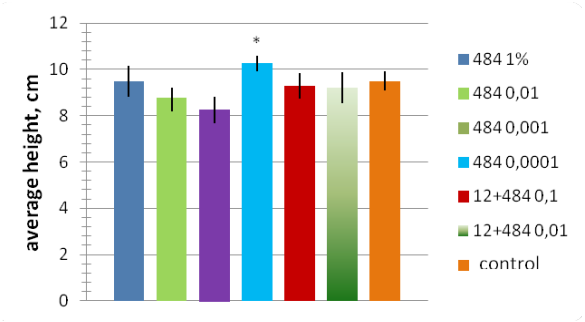


Fig. 5. Average height of wheat seedlings inoculated by bacteria and germinated in soil:
* values significantly differ from the control (P <0,05)

This coincides with the literature data: plant growth hormones, for example, auxin produced by bacilli [8] could stimulate or inhibit growth of various plant organs at different concentrations [7]. In our study, we have shown an increase in morphological characteristics of plants, which indicate the stimulation potential of the strains. The results indicate that germination of plants both in gel and in soil was most improved by bacteria of *B. megaterium* ONU 484 strain (an increase in germination of seedlings in 7.0–35.0%).

The height of seedlings and the length of roots, both in gel and in soil, were best influenced by the mixture of strains *L. plantarum* ONU 12 + *B. megaterium* ONU 484 and with bacteria *B. megaterium* ONU 484.

We gave studied the ability of bacteria to form biofilms on roots of seedlings germinated in gel with some concentrations that caused the best effect on growth characteristics.

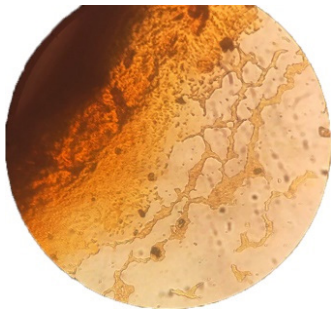
Both individual strains and strains in the mixture were able to form biofilms on roots of wheat seedlings. Some of them were represented by individual microcolonies, another were well-formed biofilms without gaps in the structure (Table 4).

Table 4

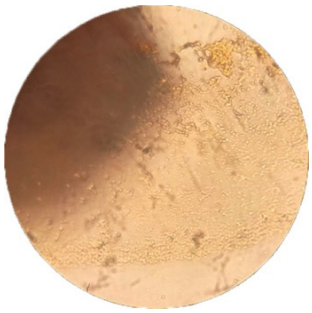
Level of biofilm formation on roots of wheat seedlings in hydroponics

Inoculum bacteria	**Concentration, cells/ml**	**Level of biofilm formation**
L. plantarum ONU 12	10^4	+++
L. plantarum ONU 12	10^3	++
B. megaterium ONU 484	10^6	++++
B. megaterium ONU 484	10^5	+++
B. megaterium ONU 484	10^4	++
B. megaterium ONU 484	10^3	++
L. plantarum ONU 12 + *B. megaterium* ONU 484	10^4	++++
L. plantarum ONU 12 + *B. megaterium* ONU 484	10^5	++++
L. plantarum ONU 12 + *B. megaterium* ONU 484	10^6	++++
L. plantarum ONU 12 + *B. megaterium* ONU 484	10^7	++++

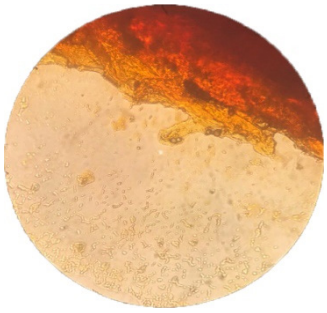
Well-formed biofilms were found on roots of the seedlings, which probably occurred due to lack of competition with other microbiota representatives and the compact gel structure that contributes to adhesion of bacteria (Figure 6).



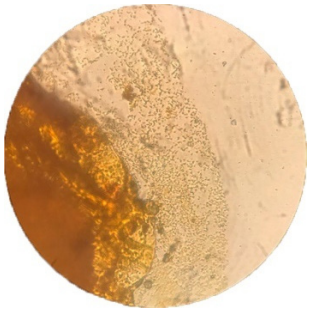
***B. megaterium* ONU 484 0.1% ("++++")**
A formed biofilm with a well-developed matrix



***L. plantarum* ONU 12 + *B. megaterium* ONU 484 0.1% ("++++")**
A formed biofilm with a well-developed matrix



***B. megaterium* ONU 484 0.0001% ("++")**
Biofilm is represented by individual entirely formed microcolonies



***L. plantarum* ONU 12 + *B. megaterium* ONU 484 0.01% ("++++")**
A formed biofilm with a well-developed matrix

Fig. 6. Biofilms of *B. megaterium* 484 and the mixture of *L. plantarum* ONU 12 and *B. megaterium* ONU 484 on roots of wheat seedlings (600x)

L. plantarum ONU 12 and *B. megaterium* 484 in the mixture formed a biofilm with a well-developed matrix regardless of the primary concentration of bacteria in inoculum. Instead, in case of the treatment with the strain *B. megaterium* 484, the level of biofilm formation depended on the concentration of bacteria and increased when higher concentrations of bacteria were applied.

The obtained results indicate that the investigated microorganisms are able to attach to roots of the seedlings, survive and form biofilms.

Conclusion

In presence of a representative of soil microbiota *B. megaterium* ONU 484 stimulation activity of lactobacilli from biological preparation increased. Strain *B. megaterium* ONU484 also showed stimulation activity when applied separately.

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ВПЛИВ БАКТЕРІЙ *LACTOBACILLUS PLANTARUM* ОНУ 12 І *BACILLUS MEGATERIUM* ОНУ 484 НА ПРОРОСТАННЯ ТА РІСТ СІЯНЦІВ ПШЕНИЦІ

Реферат

Створення біологічних препаратів для органічного землеробства має включати дослідження взаємодій мікроорганізмів – складових біопрепаратів, з представниками природньої мікробіоти агроценозів. У якості мікроорганізму – типового представника епіфітної і ґрунтової мікробіоти нами було обрано вид Bacillus megaterium. ***Мета.*** *Вивчення впливу бактерій штамів Lactobacillus plantarum ОНУ 12 і Bacillus megaterium ОНУ 484 на проростання і ріст сіянців пшениці.* ***Матеріали і методи досліджень.*** *Було проведено дослідження стимулюючих властивостей бацил штаму B. megaterium ОНУ 484, лактобацил штаму L. plantarum ОНУ 12 та їх суміші, та здатність даних мікроорганізмів до формування біоплівок.* ***Результати дослідження.*** *Показано,що за сумісної інокуляції бактеріями біологічного препарату L. plantarum ОНУ 12 та представником ґрунтової мікробіоти B. megaterium ОНУ 484 зменшення стимуляційного впливу не відбувалося. Навпаки, найбільш стимулюючий вплив на ріст рослин як в умовах гідропоніки, так і у ґрунті чинили обробки сумішшю L. plantarum ОНУ 12 + B. megaterium ОНУ 484 та окремим штамом B. megaterium ОНУ 484. У гелі середня довжина кореня сіянців збільшувалась на 8,0–16,9%, а середня висота сіянців – на 8,8–24,3%. В умовах ґрунту схожість насіння підвищувалася на 7,0%, середня висота сіянців – на 7,6%, а середня довжина кореня – на 13,1%. На корінцях пшениці лактобацили і і бацили у суміші були здатними утворювати сформовану біоплівку з добре розвинутим матриксом.* ***Висновки.*** *За присутності представника мікробіоти ґрунту B. megaterium ОНУ 484 стимуляційні властивості лактобацил біопрепарату підвищувалися. Штам B. megaterium ОНУ 484 сам по собі виявився активним стимулятором росту рослин.*

Ключові слова: *стимуляція росту рослин, мікроорганізми зі стимулювальною активністю.*

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ВЛИЯНИЕ БАКТЕРИЙ *LACTOBACILLUS PLANTARUM* ОНУ12 И *BACILLUS MEGATERIUM* ОНУ484 НА ПРОРАСТАНИЕ И РОСТ СЕЯНЦЕВ ПШЕНИЦЫ

Реферат

Создание биологических препаратов для органического земледелия должно включать исследование взаимодействий микроорганизмов –составных биопрепаратов, с представителями микробиоты агроценозов. В качестве микроорганизма – типичного представителя эпифитной и почвенной микробиоты нами было избрано вид Bacillus megaterium. ***Цель.*** *Изучение влияния бактерий штаммов Lactobacillus plantarum ОНУ 12 и Bacillus megaterium ОНУ 484 на прорастание и рост сеянцев пшеницы.* ***Материалы и методы исследования.*** *Было проведено исследование стимулирующих свойств бацилл штамма B. megaterium ОНУ 484, лактобацилл штамма L. plantarum ОНУ 12 и их смеси, и способность данных микроорганизмов к формированию биопленок.* ***Результаты исследования.*** *Показано, что при совместной инокуляции бактериями биологического препарата L. plantarum ОНУ 12 и представителем почвенной микробиоты B. megaterium ОНУ 484 уменьшения стимулирующего влияния не происходило. Наоборот, наибольшее стимулирующее влияние на рост растений как в условиях гидропоники, так и в почве вызывали обработки смесью L. plantarum ОНУ 12 + B. megaterium ОНУ 484 и отдельным штаммом B. megaterium ОНУ 484. В геле средняя длина корня сеянцев увеличивалась на 8,0–16,9%, а средняя высота сеянцев – на 8,8–24,3%. В условиях почвы всхожесть семян увеличивалась на 7,0%, средняя высота сеянцев – на 7,6%, а средняя длина корня – на 13,1%. На корнях пшеницы лактобациллы и бациллы в смеси были способными образовывать сформированную биопленку с хорошо развитым матриксом.* ***Выводы.*** *В присутствие представителя микробиоты почвы B. megaterium ОНУ 484 стимуляционные свойства лактобацилл биопрепарата повышались. Штамм B. megaterium ОНУ 484 сам по себе выявился активным стимулятором роста растений.*

Ключевые слова: *стимуляция роста растений, микроорганизмы со стимулирующей активностью.*

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