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The influence of few-layer graphene on the physiological activity of spores of the rhizosphere culture *B. Subtilis sp.*

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The article presents the results of a study of the effect of few-layer graphene obtained under conditions of self-propagating high-temperature synthesis from cellulose on the physiological activity of the rhizosphere spore culture of *B. Subtilis sp.*. It was found that few-layer graphene, as well as a dextrin/few-layer graphene composite, have a beneficial effect on the physiological activity of the rhizosphere spore culture of *B. Subtilis sp.*. After 270 days of cultivation, the number of viable cells of *Bacillus subtilis sp.* increased 4-fold in the presence of few-layer graphene, and 5-fold in the presence of a dextrin/few-layer graphene composite compared to the initial concentration. The obtained data indicate the promise of using few-layer graphene to stimulate the physiological activity of bacterial cultures, which can make few-layer graphene a useful modifying additive to agricultural soil.

Keywords: few-layer graphene, *Bacillus subtilis sp.*, soil, agricultural productivity, rhizosphere spore culture, stimulation of physiological activity.

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Introduction

The problem of hunger has not yet been solved [1,2] despite the fact that agricultural productivity has increased dramatically over the 20th century, and the requirements for agricultural productivity are increasing. Soil is a complex biological system in which various microbial communities play an important role. It is possible to radically change the characteristics of the soil by regulating the number of certain bacteria, which directly affects the final yield [3]. The use of various carbon nanomaterials, including graphene nanostructures, as modifying additives to agricultural soil is considered as one of the ways to increase agricultural productivity in this way [4]. Researchers use various types of graphene nanostructures as additives: from graphene oxide and reduced graphene oxide to graphene nanoplatelets. Although all these materials belong to the same class, but they have significant differences in chemical composition and for this reason the studies of their interaction with various microorganisms often lead to opposite results.

On the one hand, graphene oxide is known for its antibacterial properties. For instance, the authors in Ref. [5] noted that graphene oxide caused cell damage and oxidative stress in rice roots under hydroponic conditions. At the same time, the relative abundance of many endophytic bacterial communities in rice roots decreased due to the effects of graphene oxide. On the other hand, the authors in Ref. [6] noted the positive effect of graphene oxide (in concentrations up to 500 mg/l) on microbial communities of soil contaminated with cadmium. The authors also noted the impact of graphene oxide on key soil properties, namely soil pH, available potassium, phosphorus, etc. It was found in Ref. [7] that graphene has a significant effect on the number of microorganisms and the structure of the microbial community in the soil, which is clearly related to the contact time of graphene nanostructures and microorganisms. The amount of graphene of < 100 mg per kilogram of soil can increase the activity of soil microbial enzymes and bacterial biomass in a short time, which increases the rate of removal of pollutants from the soil. However, the activity of microbial enzymes and bacterial biomass in the soil is recovered over time.

The effect of graphene nanostructures on microbial communities in soil is contradictory as can be seen from the literature data. The imperfection of graphene nanostructures synthesis methods is another factor hindering their use

in agriculture. Existing methods of synthesis both using the „top–down" approach (exfoliation of graphite using surfactants and ultrasonic treatment [8], etc.) and using the „bottom–up" approach (variants of the Hummers' method [9–11], the vapor-phase deposition method [12,13]), do not allow synthesizing large volumes of material at an acceptable cost, which makes their use unprofitable. For example, the cost of reduced graphene oxide obtained by the Hummers' method can reach several million rubles per 1 km.

In our previous study, we developed a new technique for obtaining few-layer (no more than 5 layers) graphene (FLG) from biopolymers of cyclic structure under conditions of self-propagating high-temperature synthesis [14], which does not contain Stone–Wales defects [15]. It was found that FLG synthesized by this technique can be a matrix for the immobilization of oil-destroying bacteria in the creation of biological products to combat oil pollution of soil and water [16], as well as for water purification from mycotoxins [17].

The purpose of these studies is the determination of the effect of FLG on the physiological activity of rhizospheric spore culture *B. Subtilis sp.* and assessment of compatibility of FLG with this culture.

1. Experimental part

1.1. Objects of study

The rhizosphere spore culture *B. Subtilis sp.* of the root part of wheat and few-layer graphene with a specific surface area of 670 m^2/g was used as the object of the study. FLG was fabricated under conditions of self-propagating high-temperature synthesis from cellulose (microcrystalline, analytically pure, Russia). The method of synthesis of FLG is described in detail in Ref. [14].

1.2. Characterization of FLG

Electronic images of FLG were obtained by scanning electron microscopy using Tescan Mira 3-M microscope (Czech Republic) with EDX detector (Oxford instruments X-max, England). The accelerating voltage was 20 eV. The dispersion of FLG was measured by laser diffraction using Mastersizer 2000 analyzer (Malverin, USA). A plate model of particles was defined during the measurement. A suspension with a concentration of 0.05 mass.% was prepared using ultrasonic treatment in an ultrasonic bath for 5 min for measuring the particle dispersion.

1.3. Cultivation technique

Cultivation was carried out for 3.5 days at a temperature of $32 \pm 1°C$, mixing rate was 85–90 rpm. Nutrient medium composition (g/*l*): potato flakes — 7.5; peptone — 2.5; K_2HPO_4 — 1.0; $MgSO_4$ — 0.5; NaCl — 0.5; $CaCl_2$ — 0.2; $MnSO_4$ — 0.01; pH = 7.0. The culture *B. Subtilis sp.* was transferred to the spore state by heating at a temperature of 80°C after the end of cultivation. Then sterile dextrin obtained from corn starch was introduced into the culture liquid in an amount of 1% by volume of the culture liquid, mixed until a homogeneous bacterial suspension was obtained on a shaker for 20 min.

Then, a culture liquid containing a spore culture was mixed *B. Subtilis sp* with intensive stirring with FLG at the rate of 1 part FLG per 1 part of the culture fluid. At the same time, FLG was pre-sterilized under UV rays for 40 min.

The culture liquid with dextrin containing a spore culture *B. Subtilis sp*, and a culture liquid containing a spore culture *B. Subtilis sp.* in a composition with FLG was dried at a temperature of $70 \pm 1°C$, controlling the dehydration of samples by the gravitational method.

The number of viable cells (in CFU — colony-forming units) was determined using methods accepted in microbiology in the obtained samples containing culture spores *B. Subtilis sp*, dectrin and few-layer graphene [18]. The first determination of CFU is done directly after drying. Further determination of CFU was performed after storage of the samples under stable conditions at a temperature of $20 \pm 1°C$.

2. Results and discussion

Figure 1 shows SEM images of a synthesized sample of few-layer graphene.

FLG particles form aggregates with linear dimensions up to several tens of microns in powder form as can be seen from Fig. 1, *a*. However, individual particles of FLG are much smaller in size. Measurements were performed by laser diffraction for a more precise determination of the linear particle sizes (Fig. 2).

Aggregates with a size of up to several hundred microns are present in the sample as can be seen from Fig. 2 (Fig. 2, *a*). However, since the proportion of such particles is extremely small and large particles FLG form aggregates of size 0.7–0.8 μm, the signal from such particles is practically not observed in the quantitative distribution of particles (Fig. 2, *b*).

Table 1 presents the results of energy dispersion analysis. As can be seen from the table, the sample FLG has a composition typical for graphene nanostructures: an overwhelming proportion of carbon and a small proportion of oxygen associated with the end oxygen-containing groups at the edges of the sheets.

Fig. 3 shows a photo of the sample of FLG with *Bacillus subtilis sp.*

As can be seen from Fig. 3, *a*, the shape of the *Bacillus subtilis sp.* culture colonies is round with a scalloped edge, beige in color, the surface is shiny on a MPA culture medium after cultivation for 3 days at a temperature of 36°C. The diameter of the colonies is 4 mm, the number of viable cells in the control sample is $1.0 \cdot 10^4$.

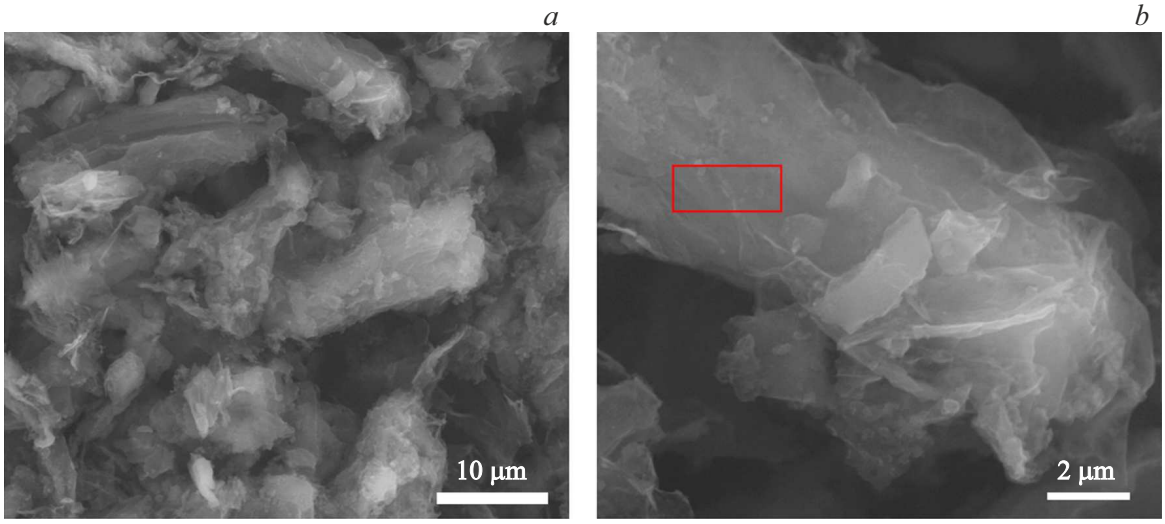

Figure 1. SEM images of FLG synthesized from cellulose; *a* — linear scale size 10 μm, *b* — linear scale size 2 μm. The red square marks the area from which the signal was taken during the energy dispersion analysis.

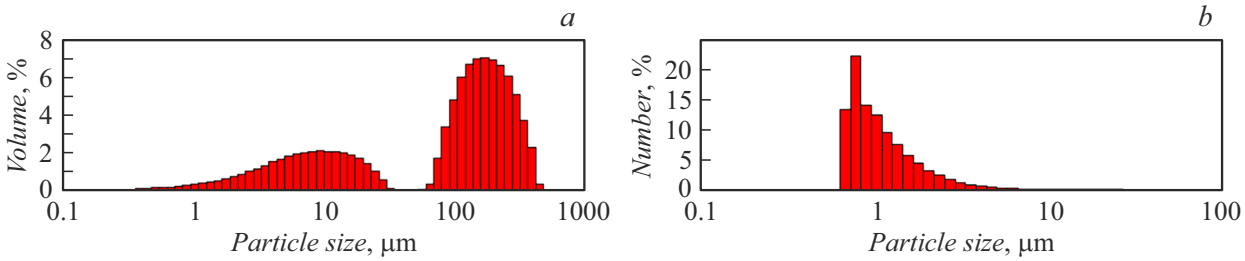

Figure 2. Distribution of FLG particles by volume (*a*) and by number of particles (*b*).

Table 1. Results of the elemental analysis of a FLG sample from cellulose

Element	Weight percentage	Atomic percentage
Carbon	94.5 ± 0.2	95.8 ± 0.2
Oxygen	5.5 ± 0.2	4.2 ± 0.2

At the same time, the shape of *Bacillus subtilis sp.* culture colonies on a MPA nutrient medium also remains round with a scalloped edge during cultivation for 3 days at a temperature of 36°C after injection of FLG into the culture liquid and storage for 270 h (Fig. 3, *b*). The color of the colonies is from light gray to dark gray. Colonies with a diameter of 1 to 4 mm are observed. The shape of *Bacillus subtilis sp.* culture colonies on a MPA nutrient medium during cultivation for 3 days at a temperature of 36°C after injection of the dextrin/FLG composite into the culture liquid and storage for 270 h (Fig. 3, *c*) also remains round with a scalloped edge. The color of the colonies is from light gray to dark gray. Colonies with a diameter of 0.5 to 4 mm are observed.

The analysis of the shape of colonies of *Bacillus subtilis sp.* culture shows that few-layer graphene significantly affects the form of existence of *Bacillus subtilis sp.* culture cells— the shape of the colony, the size, the nature of the edges and surface, as well as the color, which is due to the black color of graphene. Few-layer graphene increases the physiological activity of it Bacillus subtilis sp. culture in combination with dextrin

Table 2 shows the results of measuring the concentration of *Bacillus subtilis sp.* spores with FLG and with the dextrin/FLG composite depending on the duration of the experiment.

As can be seen from Table 2, the concentration of *Bacillus subtilis sp.* increased by 4 times in the presence of FLG on 270 day of observations, and it increases by 7 times compared to the initial concentration in the presence of a dextrin/FLG composite.

It should be noted that graphene nanostructures can exhibit both probiotic and antibacterial properties, depending on the parameters of the graphene nanostructures themselves. Usually, graphene nanostructures containing a large amount of oxygen in their composition exhibit antibacterial properties, primarily — graphene oxide [19]. The antibacterial properties of such graphene nanostructures

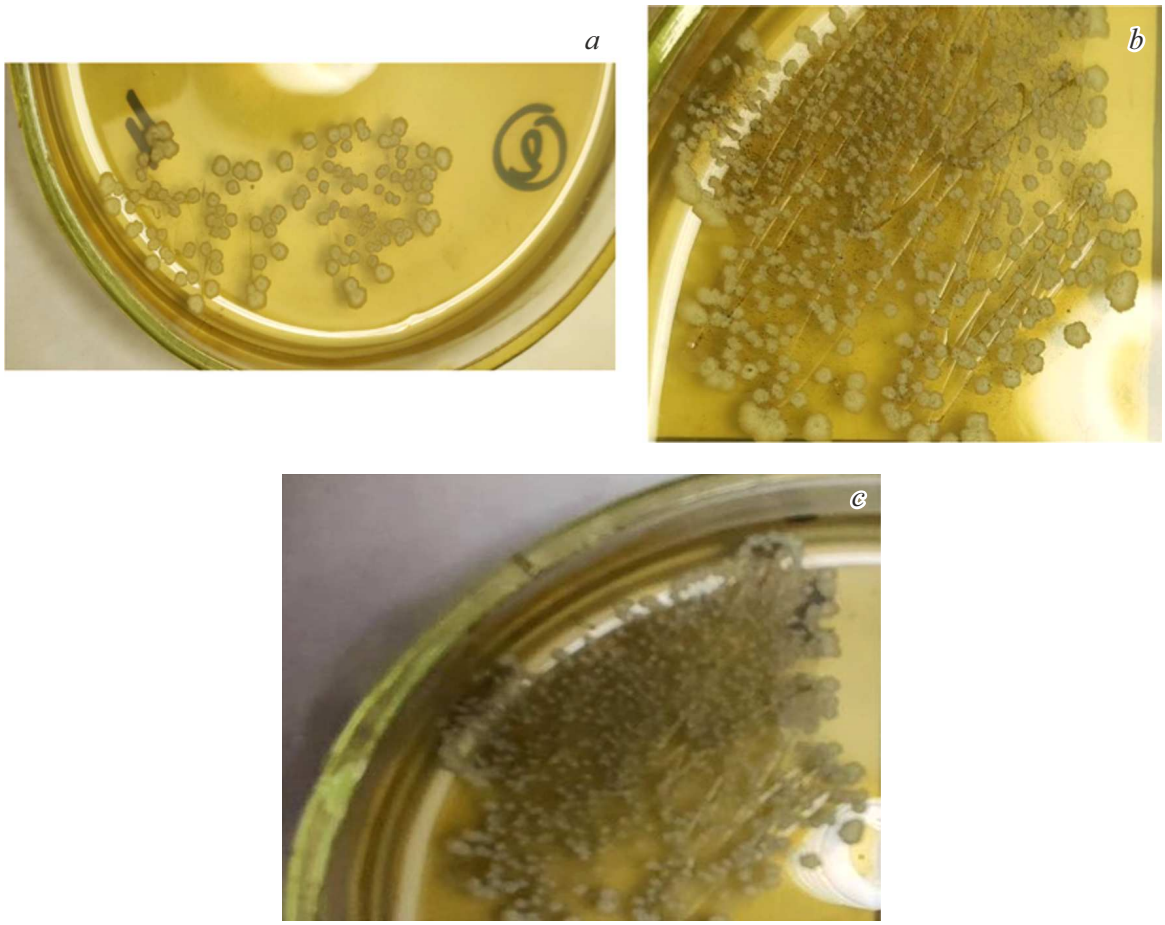

Figure 3. Appearance of the FLG sample with *Bacillus subtilis sp.*: *a* — shape of *Bacillus subtilis sp.* culture colonies after cultivation; *b* — shape of *Bacillus subtilis sp.* culture colonies after injection of FLG into culture liquid and storage for 270 h; *c* — shape of *Bacillus subtilis sp.* culture colonies after the injection of the dextrin/FLG composite into the culture liquid and storage in 270 days,h.

Table 2. Effect of FLG nanoparticles on the physiological activity of *Bacillus subtilis sp.* spores in compositions with dextrin and without dextrin

Culture	Presence of FLG	Presence of dextrin	Initial concentration, CFU/m*l*	Concentration after 30 days, CFU/m*l*	Concentration after 150 days, CFU/m*l*	Concentration after 270 days, CFU/m*l*
Bacillus	1 to 1	–	$1.0 \pm 0.2 \cdot 10^4$	$3.0 \pm 0.2 \cdot 10^4$	$3.3 \pm 0.2 \cdot 10^4$	$4.0 \pm 0.2 \cdot 10^4$
subtilis	1 to 1	1 vol.%	$1.0 \pm 0.2 \cdot 10^4$	$4.2 \pm 0.2 \cdot 10^4$	$6.0 \pm 0.2 \cdot 10^4$	$7.0 \pm 0.2 \cdot 10^4$
sp.	1 to 1	1 vol.%	$1.1 \pm 0.2 \cdot 10^4$	$1.3 \pm 0.2 \cdot 10^4$	$1.8 \pm 0.2 \cdot 10^4$	$1.9 \pm 0.2 \cdot 10^4$

are attributable to two main mechanisms: the death of pathogen cells due to oxidative (oxidative) stress, primarily as a result of the formation of reactive oxygen species (ROS), and damage to the membrane of pathogen cells by structural defects of graphene nanostructures (primarily by sheet edges) [20]. The first mechanism is directly related to the oxygen content in the particles of graphene nanostructures. That is why graphene oxide, in which the proportion of oxygen reaches 30–40 at.%, exhibits high antibacterial properties. The second mechanism is related to the particle dispersion of graphene nanostructures. The authors experimentally showed in Ref. [21] that more highly dispersed graphene nanostructures have greater antibacterial efficacy compared to low-dispersed ones.

The oxygen concentration is relatively small (4.5,at.%) in FLG used in this study, and the linear particle sizes can reach several tens of microns. Therefore, FLG particles can act as colony-forming centers, which increases the physiological activity of spores of *Bacillus subtilis sp.*

Conclusion

It was found that few-layer graphene contributes to the preservation and increase of the physiological activity of the spores of the *Bacillus subtilis sp.* culture, which indicates its compatibility with this culture. The use of few-layer graphene together with dextrin made it possible to further increase the physiological activity of spores in comparison with pure few-layer graphene. The data obtained indicate the high prospects of using few-layer graphene to increase the activity of the necessary bacterial cultures, which can make few-layer graphene a useful modifying additive in agricultural soil.

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Conflict of interest

The authors declare that they have no conflict of interest.

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