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Study Of Antagonism Of Endophytic Bacterial Isolates Against Fusarium Sporotrichioides

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ABSTRACT

Field isolates of micromycetes of the genus Fusarium are widespread in natural biotopes and can synthesise metabolites that are toxic to animals and humans, the most aggressive of which are trichothecene mycotoxins. The Fusarium sporotrichioides micromycete is the most pathogenic for cereals and vegetable crops, leading to yield loss and soil contamination. The metabolites T-2 toxin and zearalenone it produces have high carcinogenic and general toxic effects. For that reason it is necessary to carry out mycological monitoring of cereal crops, products of their processing, crop products in order to prevent the spread in nature of fungus F. sporotrichioides, contamination by it and its metabolites of feed raw materials, toxicosis in animals, and humans, having a devastating effect on the body, causing violations of immune, reproductive and other systems, low productivity, increased mortality in young animals, predisposition to infectious diseases. In addition, an urgent issue today is the development of alternatives to chemical means of biological plant and feed protection against pathogenic musty microorganisms. In this work, we isolated the microscopic mold fungus F. sporotrichioides from wheat grain, formed a collection of isolates of endophytic microorganisms, and studied their antagonistic activity. Some of the isolates showed a fungistatic antibiotic type of antagonism, they inhibited the growth of the mycelial fungus at a distance under the influence of the produced antibiotic substances, with the formation of a "sterile" zone between the cultures. Some endophytic isolates were characterized by fungistatic alimentary antagonism, which is expressed in stopping the growth of the fungus upon contact with the colony of the antagonist. It was found that the isolates produced metabolites to varying degrees that have a fungistatic effect. According to the results of experiments in vitro and in vivo, the most technologically advanced and safe isolates were selected, promising for the control of phytopathogenic microscopic fungi F. sporotrichioides. The combination of tinctorial and morphological features, results genetic confirmation by amplification of the sod - superoxide dismutase gene made it possible to assign endophytic microorganisms to the species Bacillus subtilis.

Keywords: endophytic bacteria, Fusarium sporotrichioides, antagonism, enzymatic activity, safety, Bacillus subtilis.

INTRODUCTION.

Problems in ensuring food security are microscopic mold fungi that contaminate food and feed raw materials from the moment of harvesting to processing and during storage. Under favorable conditions, these micromycetes synthesize highly toxic metabolites, many of which have cytotoxic, mutagenic, carcinogenic, immunosuppressive, and teratogenic properties (Ekblad et al., 2013; Gindullin et al., 2015; Kadikov et al., 2018; Zheng et al., 2019; Potekhina et al., 2020). Isolates of Fusarium fungi were isolated from samples of feed and agricultural products from various regions of the Russian Federation, and it was found that they had a high toxigenic potential. Microscopic fungi reduce the sowing quality of seeds, inhibit plant development, and reduce the nutritional quality of crop products, so they are considered the most harmful pathogens of crop diseases (Sekomo et al., 2012; Hujslova et al., 2017).

Antagonist bacteria represent an effective and safe alternative to chemical agents for controlling plant and feed phytopathogens as biological defense agents, since they effectively restrain the development and spread of pathogens, have a beneficial effect on microbiocenoses, and stimulate plant growth (Beneduzi et al., 2012; Choudhary and Johri 2009).

Improving the methods of biological control and containment of pathogens is necessary in modern conditions and is feasible by isolating new microorganisms that exhibit antagonistic properties (Mardanova et al., 2017).

The aim of the study was to search for endophytic bacteria that exhibit antagonistic activity against the toxigenic causative agent of Fusarium sporotrichioides.

Materials and Methods. Endophytic bacterial isolates selected from the vegetative parts of wheat, sunflower, and maize were the subjects of the study. The plants were collected during the ear emergence phase, when the rhizosphere microbiota was most active. The material was washed three times with distilled water, cut into fragments, the ends of which were treated with paraffin and then sterilised (Hallmann and Berg, 2006, Qin et al., 2009). The sterility control was then performed by placing a portion of the samples on nutrient media for the cultivation of bacteria and fungi and incubated under thermostatic conditions under appropriate regimes. After obtaining a negative result, the second part of the samples was sterilely ground, filled with sterile distilled water, then the suspension was sown into Petri dishes on the surface of GRM-agar (Federal Budget Institution of Science "State Research Center for Applied Microbiology & Biotechnology", Obolensk, Russia) followed by cultivation at 37^0C for 24 hours. Pure cultures of isolates were obtained by reseeding individual colonies.

The antagonistic properties of endophytes were evaluated against a field isolate of the micromycete Fusarium sporotrichioides isolated from the surface of wheat seeds. The phytopathogen was cultured on Chapek's

medium at 26^0C. The identification of the fungus was based on phenotypic features, the species identity was confirmed by amplification with species-specific primers complementary to the sequence (GenBank ID: MN452662. 1) encoding ITS (internal transcribed spacer) 1, ITS2, and a large subunit of ribosomal RNA, the specificity of this sequence for the indication of Fusarium sporotrichioides was confirmed by analysis of possible homologies in various organisms, in the BLASTn utility (Zheng et. al, 2000), according to which more than 1000 nucleotide sequence matches with the Fusarium sporotrichioides genome were identified.

Nucleic acid extraction was performed using the "MAGNO-sorb" kit according to the manufacturer's instructions.

The composition of the reaction mixture for PCR (polymerase chain reaction) amplification was as follows, per sample: 1.5 µl 25 mM $MgCl_2$ solution; 1.5 µl 2.5 mM dNTP solution; 1.5 µl 10x PCR buffer; 0.5 µl 10 pM PCR probe solution (5`-R6G-cccgccgcaggaaaccctaaactct-BHQ1-3`); 10 pM solution of forward (5`-ttgcctcggcggatcagc-3`) and reverse (5`-tccgttgttgaaagttttgatttatttgtt-3`) primers; 0.5 µl each; 0.5 µl Taq polymerase; 5 µl DNA and 3.5 µl deionized water. For PCR, we used reagents manufactured by CJSC Syntol, Moscow, Russia. Plasmid DNA pAL2-T (ZAO Evrogen, Moscow, Russia) containing marker DNA was used as a positive amplification control, having the following nucleotide sequence "5`-ttgcctcggcggatcagcccgcgccccgtaaaacgggacggcccgccgcaggaaaccctaaactctgtttttagtggaacttctgagtataaaaaacaaataaatcaaaactttcaacaacgga-3`".

PCR was performed on a C1000 amplifier with a CFX96 optical unit (BioRad). The programme for the amplification was as follows: (I) DNA denaturation at 95 °C for 2 minutes; (II) 5 cycles consisting of: 10 seconds at 95 °C, 30 seconds at 59 °C; (III) 40 cycles consisting of: 10 seconds at 95 °C, 30 seconds at 59 °C, detection of PCR result (fluorescence) occurs on each of the 40 cycles of the third PCR stage, at 59 °C. The amplification result of the tested micromycete colonies is shown in figure 1.

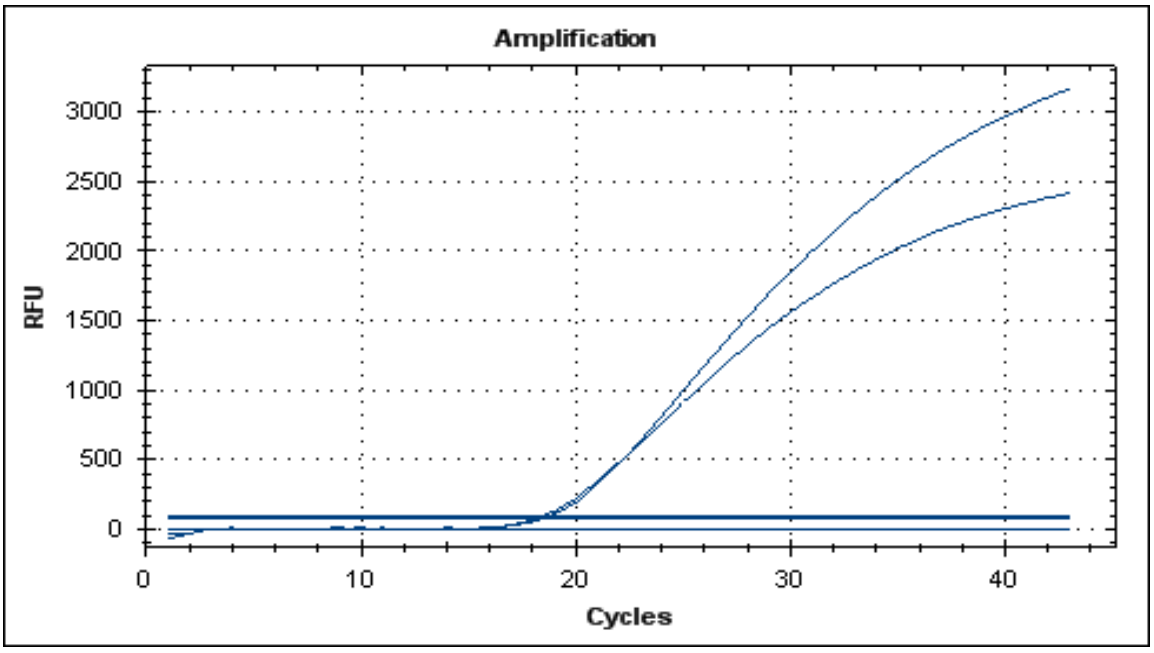

Figure 1: Indication of genetic material of Fusarium sporotrichioides

The amplification of the test materials revealed an accumulation of fluorescence, through the R6G channel, starting at cycle 24, indicating the presence of Fusarium sporotrichioides DNA in the sample.

The antagonistic activity of the bacterial isolates was determined by the counterculture, agar block and modified streak methods.

In a double-culture experiment, the micromycete and antagonist isolates were grown separately on agarised media under appropriate conditions. A block with mycelium of the fungus was then cut out with a sterilised drill and placed on the surface of the medium in a Petri dish, stroking the antagonist culture at a distance of 6 cm from it. A micromycete sowing without the isolate served as a control. The dishes were incubated in an incubator at 26^0C, recording the experiment at 5; 10; 15 and 20 days of cultivation, noting the growth of micromycete and antagonist, the nature of their interaction, the zone of inhibition of micromycete (Montealegre et al., 2003).

The degree of inhibition of pathogen mycelial growth was determined using the formula:

Inhibition (%) = [(R - r)/R×100],

where R - radial growth of the fungus in the control;

r - radial growth of the fungus in the presence of bacteria.

The agar block method consisted of culturing a bacterial isolate on a dense nutrient medium in a Petri dish until a "solid lawn" was formed, followed by cutting out blocks with a sterile cork drill and transferring them to a test fungus culture pre-seeded wort agar (CJSC "NITSF", St. Petersburg, Russia) surface in another Petri dish. The test culture was inoculated with a spatula and the agar blocks were placed upwards at an equal distance from each other and from the edge of the dish, pressing them firmly against the surface of the medium. The control was a cultivation of a micromycete without an antagonist. The number of repetitions in each group corresponded to 4. The dishes were incubated in a thermostat at 26^0C. The experiment was recorded on days 2; 4 and 7. After 7 days of cultivation, the diameter of the mycelial growth suppression zone was measured in two mutually perpendicular directions. The activity of the antagonist bacteria was calculated according to the formula:

A=D/d,

where D - diameter of the mycelial growth retardation zone of the fungus, mm;

d - diameter of the crop application site, 13 mm

The modified stroke method. The test fungus culture was grown in potato broth at 26^0C, stirring constantly at 90 rpm for 7 days. The melted beef-extract agar was poured into sterile Petri dishes and a 1 cm wide strip was cut out with a sterile scalpel along the diameter of the dish after freezing. The molten and cooled to 45^0C wort agar with test-fungus filtrate was then poured into the formed trough. After solidification of the medium, the test isolates were stroked across the entire area of the dish, crossing in a perpendicular direction to the groove. The control was a dish with a test culture without isolates. The dishes were incubated in an incubator at 26^0C for 7 days. The result was accounted for by the development of the test fungus.

The bacterial isolates were cultured in GRM broth (State Research Center for Applied Microbiology and Biotechnology, Obolensk, Russia) under thermostat conditions at 37^0C for 72 hours. The contents of the containers were then centrifuged under sterile conditions at 13,000 rpm for 30 min, the supernatant was separated and filtered through Millipore 0.22 µm membrane filters (CAMEO, GVS, Italy). The filtrate was added to Chapek's agarized medium melted and cooled to 45^0C in a 1:4 ratio and poured into sterile Petri dishes, then after solidification, a micromycete suspension at a concentration of 1×10^3 spores/ml was applied to the medium surface and spread evenly using a microbiological spatula. The control was dishes with test fungi without filtrates. The number of repetitions in each group was 5. The plates were incubated at 26^0C for 7 days and the colonies grown were counted.

The fungistatic activity of metabolites of endophytic microorganisms was assessed by the degree of inhibition of pathogen mycelial growth:

% inhibition = (1 - (Fungal growth/Control growth)) x 100,

where Fungal growth - number of colonies in the experiment;

Control growth - number of colonies in the control.

The enzymatic activity of the antagonist bacteria was assessed for amylolytic, proteolytic, lipolytic, cellulolytic properties and the ability to produce catalase and chitinase.

The amylolytic activity of microorganisms was determined by culturing them in a starch-containing medium (the following composition: 10 g peptone, 5 g KH_2PO_4, 2 g starch, 15 g agar-agar per 1 L of distilled water). After autoclaving at 120^0C for 30 minutes, the medium was poured onto sterile Petri dishes and the test microorganism was inoculated onto the solidified surface of the medium by stroking the diameter of the dish, the control being the dish with the medium. All the dishes were placed in a thermostat at 37^0C. After 4 days, the dishes were removed from the thermostat and 3 ml of Luegol solution was added to each cup. In the presence of starch, the medium stained intensely blue, indicating the absence of amylolytic activity in the tested isolate. When starch hydrolysis capacity was present, the medium was stained yellow-brown. The result of the test was judged by the character of the staining of the medium.

The presence of lipolytic activity in the isolates was judged by acidification of the medium (Stern broth) during cultivation. The broth was prepared as follows: 50 drops of saturated alcoholic solution of basic fuchsin, 10 ml glycerol, and 20 ml of freshly prepared 10% sodium sulphate solution were added to 1 l of meat-peptone broth. Olive oil was added to the prepared broth at the rate of 1 ml per 100 ml of broth. The medium was then poured into 10 ml tubes each and 100 µl of the test culture suspension was added. Tubes were placed in a thermostat at 37^0C for 120 hours while the pH of the broth was measured on days 1, 3, and 5. A sterile nutrient medium without microorganisms was used as a control.

Proteolytic activity was determined by seeding cultures on Eikman's milk medium. For that purpose, 200 ml of sterile, skimmed milk was added to 1 litre of melted meat-peptone agar, stirred until homogeneous, and poured into sterile Petri dishes. After solidification of the medium, 200 µl of daily culture suspension was added to each dish, spread over the surface, and placed in a thermostat at 37^0C. After 3 days, the results of the test were recorded by the appearance of lumen zones. In the presence of proteolytic activity in the isolate under study, a lucent zone was formed around the growing culture, which stood out clearly against the overall cloudy background of the medium.

The cellulolytic activity of the isolates was determined by culturing them on starvation agar with a piece of sterilised filter paper weighing 60 mg as a carbon source. The culture was inoculated on a frozen agar surface as a suspension on physiological solution (300 µl) and cultured at 37^0C. At 30 days of the experiment, the paper was removed, washed from the agar, dried, and weighed. The difference in the weight of the filter paper at the beginning and the end of the study indicated the cellulose degradation capacity of the cultures tested.

The ability of the microorganisms to synthesise catalase was assessed by adding 1 ml of a 3% hydrogen peroxide solution to a test tube containing a culture grown on GRM-broth. The positive reaction to the enzyme was judged by the formation of a "foam cap".

To determine the chitinolytic activity of isolates the following medium was used (g/l): sucrose - 20.0; $NaNO_3$ – 3,0; KH_2PO_4 – 1,0; $MgSO_4$ - 0.3; chalk - 10.0; agar - 20.0; distilled water - up to 1 l. Isolates were hatched on medium and incubated for 7-14 days. A positive result for chitinolytic activity was the formation of lumen zones around the microbial colony.

A prerequisite for the suitability of micro-organisms for the production of biopreparation is the safety of the feed treated with them and the absence of pathogenic properties of the micro-organisms included in their composition.

The toxicity of wheat grain infected with Fusarium sporotrichioides and treated with the most effective isolates at a titer of 1×10^{11} CFU/ml was determined in experiments on white mice and protozoa. Toxicological evaluation of microorganisms at 1×10^{11} CFU /ml titer was carried out by determining toxicity, virulence and toxigenicity parameters on white mice and rats.

Isolates were identified by morphological and cultural features, and species identity was confirmed by polymerase chain reaction using genospecific primers. The methodology for genetic identification of the putative microbial species was identical to that described above (section 2. 1), the difference was in the oligonucleotides used (PCR probe 5`-Rox- tgctttagaaccgcatatcgacaaggaaact-BHQ2-3`, forward primer 5`-gcttacgaacttccagaattaccttatgc-3`, reverse primer 5`-gtgttgtggtgtttcgtatggtgaatagt-3`). Plasmid DNA pAL2-T (Eurogen, Moscow, Russia) containing marker DNA with the following nucleotide sequence "5`gcttacgaacttccagaattaccttatgcgtacgatgctttagaaccgcatatcgacaaggaaactatgactattcaccatacgaaacaccacaacac-3`" was used as a positive amplification control.

We relied on Statistica 6.0 for the statistical processing of the obtained results. Two-sample Student's t-test was used to compare data. We set the critical level of statistical significance (p) at 0.05.

Results and Discussion. Based on the results of the initial screening from the vegetative parts of wheat, sunflower, and maize, 18 bacterial isolates were isolated, which have been conventionally labelled as EFS.

Based on the mechanism of antifungal action, the endophyte isolates were divided into those with a high motility index and those forming a sterile antagonistic zone, based on the evaluation of the results of the counter-culture experiment. Among the isolates with motility (Table 1), high inhibitory activity against the micromycete F. sporotrichioides was shown by EFS3, EFS10, EFS14, and EFS15; at 10 days of co-culture they occupied the largest area of nutrient substrate of Petri dishes, blocking further growth of test culture fungus, near the growth zone mycelium of pathogen was partially lysed. Isolate EFS5 occupied half of the dish area by 30 days of incubation, the development of isolates EFS2, EFS7, EFS12 and EFS16 was inhibited by the test micromycete, proliferating in the area they occupied.

Table 1. Growth of F. sporotrichioides mycelium from blocks with isolates of endophytic microorganisms with a high motility index, mm

Isolate	The term of joint cultivation of the isolate and micromycete			
	5 days	10 days	15 days	30 days
EFS2	26,00 (2,24)	31,20 (2,17)	37,80 (3,70)	45,30 (3,03)
EFS3	20,10 (2,56)	21,20 (2,49)	24,50 (2,78)	26,00 (4,18)
EFS5	27,20 (2,59)*	29,40 (2,70)	32,20 (1,92)	35,20 (2,59)
EFS7	28,20 (2,59)*	35,10 (2,66)	42,20 (3,56)	50,40 (3,29)
EFS10	16,00 (2,00)	17,40 (2,95)	18,90 (2,41)	19,50 (1,94)
EFS12	27,80 (2,59)*	33,20 (2,95)	38,80 (3,03)	46,60 (1,82)
EFS14	14,80 (1,30)	15,30 (1,57)	17,20 (2,28)	19,00 (1,87)
EFS15	16,00 (1,87)	20,20 (2,28)	22,20 (1,30)	26,20 (2,28)
EFS16	23,00 (2,12)	29,40 (2,07)	36,00 (2,24)	44,70 (2,17)
F. sporotrichioides	31,10 (2,46)	40,00 (2,24)	47,80 (3,03)	67,00 (3,32)

Note. The table shows the average values for groups in the format M (Sd), where M - arithmetic mean; Sd - standard deviation. * - differences with the group of F. sporotrichioides (control) are not reliable

Sterile zone width from 16.9 to 22.5 mm between the isolates and the micromycete celebrated for 10 days of joint cultivation, but on the 15th day of the experience of distance between pathogen isolates and EFS6, EFS11 and EFS18 were noted, the zone of inhibition of the mycelium of the fungus remaining isolates was 3.2 – 19,5 mm. Isolates EFS8, EFS9, EFS13, and EFS17 retained the sterile zone even by 20 days (Table 2).

Table 2. The width of the zone of inhibition of the growth of F. sporotrichioides by isolates of endophytic microorganisms with an antibiotic type of antagonism, mm

Isolate	The term of co-cultivation of the isolate and micromycete		
	10 days	15 days	20 days
EFS1	22,20 (1,92)	3,50 (1,41)	0, 00 (0,00)
EFS4	21,10 (1,95)	5,40 (2,51)	0, 00 (0,00)
EFS6	17,30 (1,99)	0,00 (0,00)	0, 00 (0,00)
EFS8	19,68 (3,39)	10,20 (1,92)	3,90 (2,46)
EFS9	18,20 (2,28)	18,80 (2,17)	17,30 (2,73)
EFS11	21,00 (2,92)	0,00 (0,00)	0, 00 (0,00)
EFS13	21,20 (1,79)	19,10 (2,25)	11,80 (2,59)
EFS17	20,40 (2,88)	15,50 (2,78)	10,90 (2,41)
EFS18	20,10 (0,74)	0,00 (0,00)	0, 00 (0,00)

Note. The table shows the average values for groups in the format M (Sd), where M - arithmetic mean; Sd - standard deviation. * - differences with the group of F. sporotrichioides (control) are not reliable

By the method of agar blocks, the growth delay zone of the micromycete was established in 11 isolates from 18 selected ones. The EFS9 culture formed the largest zone of growth retardation of the micromycete (M = 27.00 mm, Sd = 1.00 mm), its antagonistic activity was the highest compared to the others and amounted to 2.01±0.18 (M± Sd). The retention zone of the mycelium of the fungus F. sporotrichioides when co-cultured with the EFS14 isolate was 25.89 mm (Sd = 0.82 mm), the activity was 1.81±0.14. In the remaining isolates, the fungal growth retardation zone and antagonistic activity were, respectively: EFS10 - 24.50±0.70 mm and 1.81±0.14, EFS13 - 23.20±0.70 mm and 1.72±0.16, EFS15 - 22.70±0.70 mm and 1.68±0.18, EFS17 - 20.00±1.00 and 1.48±0.24, EFS3 - 15.70±0.70 and 1.16±0.14, EFS4 - 14.25±0.38 and 1.06±0.08. Isolates of endophytic bacteria with the codes EFS 2, EFS5, EFS6, EFS11, EFS12, EFS16 and EFS18 did not show antagonistic activity to the test culture in the agar block method.

Using the modified stroke method, we found that the isolates isolated to a greater or lesser extent also had antagonistic activity against the test culture fungus, the pathogen growth retardation zone by 7 days of co-culture ranged from 3.6 mm to 15.5 mm (Figure 2). The EFS3, EFS10, EFS14, and EFS15 isolates were the most active, their zone of growth retardation of F. sporotrichioides being 14.17 mm (Sd = 0.86 mm), 13.17 mm (Sd = 0.66 mm), 14.50 mm (Sd = 0.50 mm), and 15.17 mm (Sd = 0.66 mm), respectively.

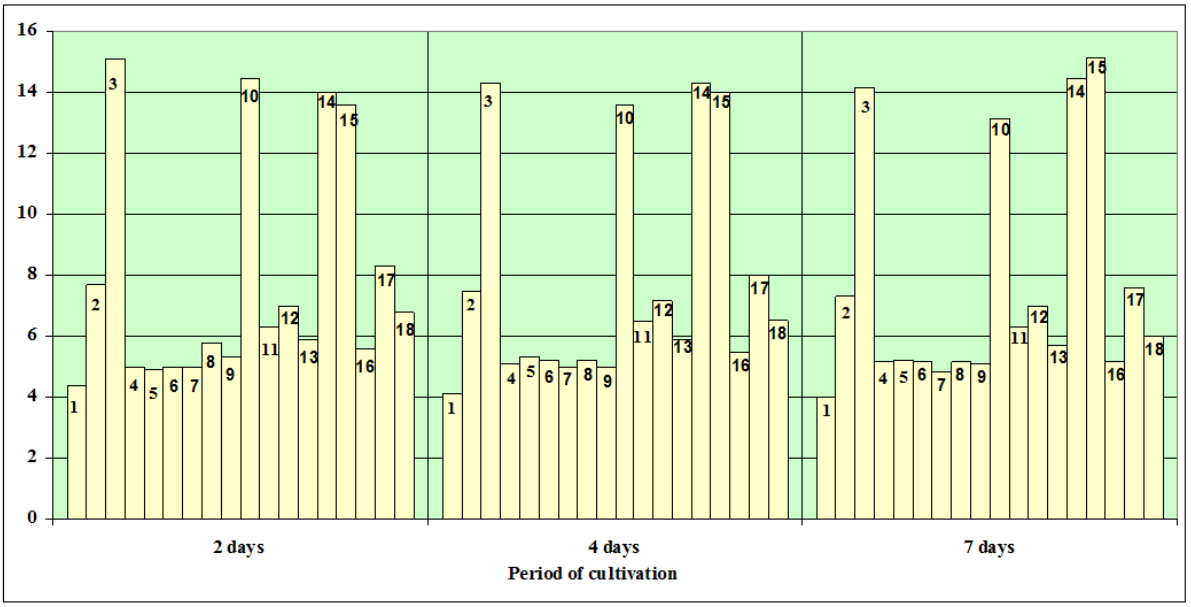

Figure 2. The zone of growth retardation of F. sporotrichioides by isolates of endophytic microorganisms, determined by the stroke method, mm (Note: 1-EFS1, 2-EFS2, 3-EFS3, 4-EFS4, 5-EFS5, 6-EFS6, 7-EFS7, 8-EFS8, 9-EFS9, 10-EFS10, 11-EFS11, 12-EFS12, 13-EFS13, 14-EFS14, 15-EFS15, 16-EFS16, 17-EFS17, 18-EFS18)

In the delayed antagonism experiment, the fungistatic effect of metabolites produced by the isolates was evaluated by counting the number of grown colonies and expressed by the degree of inhibition of micromycete growth (Figure 3).

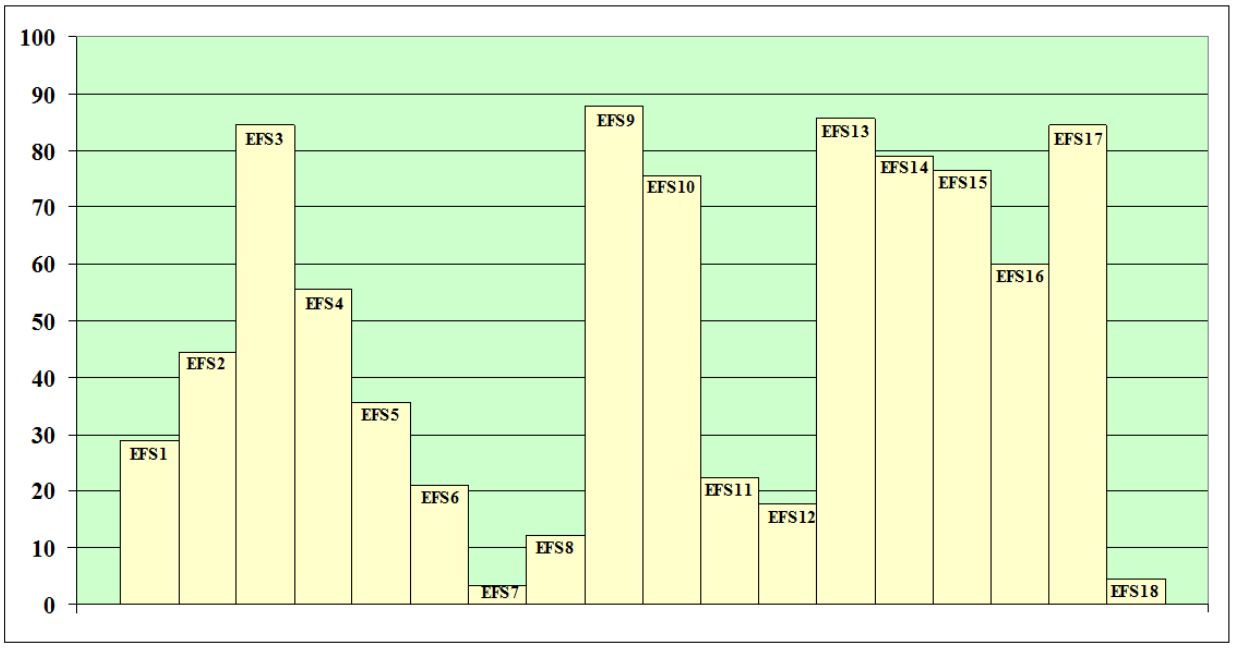

Figure 3. Fungistatic activity of metabolites of endophytic bacterial isolates against the micromycete F. sporotrichioides, % inhibition

The degree of inhibition of fungus development by metabolites of isolate EFS9 was the highest, the activity of EFS3, EFS13, and EFS17 was lower by 3.43, 1.03, and 3.34%, respectively, the efficiency of EFS10 in comparison with the most active isolate was lower by 12.22%, EFS14 was 8.89%, EFS15 was 11.11% and EFS16 was 27.78%. The EFS7, EFS18, EFS8, EFS12, EFS11, EFS6, and EFS1 isolates had minimal fungi static activity, the degree of inhibition of pathogen development by the remaining isolates being 35-45%.

As a result of the conducted studies, it was found that the active isolates are detected using each of the methods. At the same time, the formulation of several complementary experiments allowed us to better assess the nature of the interaction between the antagonist and the pathogen. It was found that the isolates of endophytic bacteria EFS3, EFS9, EFS13 and EFS17 had the greatest antifungal potential against F. sporotrichioides. They were characterized by alimentary and antibiotic types of antagonism and therefore they were selected for further research.

Morphologically, the isolates were characterized as gram-positive, immobile bacilli capable of forming spores. When cultured in liquid media (meat-peptone agar), they formed flat, opaque, shiny, beige, or white colonies with jagged edges; when cultured in liquid media (GRM-broth), they formed a film on the surface.

Hydrolases synthesized by microorganisms promote their absorption of nutrients. Most of these enzymes belong to the category of inducible and are synthesized in response to the presence in the environment of the substrate-inducer necessary for the cell. Competition for nutrients becomes a factor in suppressing the germination of phytopathogen spores when there is an excess of nutrients and microorganisms in the environment. The antagonistic properties of bacteria and their ability to lysis can be manifested with a small number of microorganisms and a lack of nutrients. In this case, the complex action of various hydrolytic enzymes will be a condition for the effective lysis of pathogenic fungi and / or the use of fungal mycelium as a food source.

The isolates of endophytic bacteria selected by us, which showed the greatest antifungal effect against F. sporotrichioides, also had a high level of enzymatic activity. All isolates were characterized by the presence of amyl lytic activity, this was evidenced by the staining of the nutrient medium on which they were cultured, in yellow-brown color when adding a Lugol solution. During the cultivation of EFS9, EFS13, and EFS17 isolates in Stern broth, no significant acidification of the medium was recorded, which indicated a moderate lipolysis activity characteristic of these bacteria. On the Eijkman milk medium, the formation of zones of enlightenment

around the grown colonies of the studied microorganisms was noted, which clearly stand out against the general cloudy background of the medium, this circumstance allows us to conclude that the isolates have proteolysis activity. The EFS3 isolate was characterized by the presence of cellulolytic activity, which was established by the difference in the mass of the filter paper at the beginning and end of the study after 30 days of cultivation on a starved agar medium, therefore, the microorganism used cellulose as a food source, which was hydrolyzed by the corresponding enzymatic complex produced. The other isolates did not have this ability. When growing each of the tested microorganisms on a GRM broth and then adding a solution of hydrogen peroxide to the medium, the formation of a "foam cap" was noted, this is evidence of the ability of the isolates to synthesize catalase. The isolates EFS3, EFS9 and EFS13 also had the ability to produce chitinase, which was established by the formation of zones of enlightenment around the colonies of microorganisms when cultured on a nutrient medium of a certain composition.

Subsequently, the grain contaminated with the fungus F. sporotrichioides was treated with a suspension of each of the selected isolates. In the extract of grain samples affected by the test microscopic fungus, the survival rate of infusoria Stylonychia mytilus was 42.84% (Sd = 5.26%), in the extract of toxic grain after treatment with EFS17 isolate - was lower in comparison with the control (92.24±3.00, M±Sd) by 21.59%, and endophytic bacteria under the codes EFS13 (83.40±3.68, M±Sd), EFS3 (84.64±4.22, M±Sd) and EFS9 (86.14±3.94, M±Sd), respectively, by 8.84, 7.60 and 6.10 %.

When parenteral administration of a grain extract contaminated with F. sporotrichioides to white mice, as well as an extract of fusarium grain treated with a suspension of EFS17 isolate, the death of experimental animals was noted; this indicated the presence of toxic properties in the grain and the non-applicability of this isolate in practice. Treatment of toxic feed with suspensions of other endophytic bacteria had a detoxifying effect on it and when the solution was administered intraperitoneal, it did not lead to the death of mice; when opening animals killed by decapitation, no visible changes were observed in the internal organs.

According to the results of toxicological evaluation of bacterial isolates EFS3, EFS9 and EFS13 in experiments on white mice and rats, no visible changes in the clinical status and death of animals were found during intraperitoneal and oral administration of the material. Thus, the studied microorganisms do not have toxic, virulent and toxigenic properties.

The antagonist bacteria under study were identified as Bacillus subtilis by cultural and morphological features, genetic confirmation was done by amplification of the gene sod - superoxide dismutase (GenBank ID: EF203905.1). The amplification of this gene allows the identification of up to 199 isolates/strains belonging to the species Bacillus subtilis.

The result of amplification of the sod - superoxide dismutase gene is shown in Figure 4.

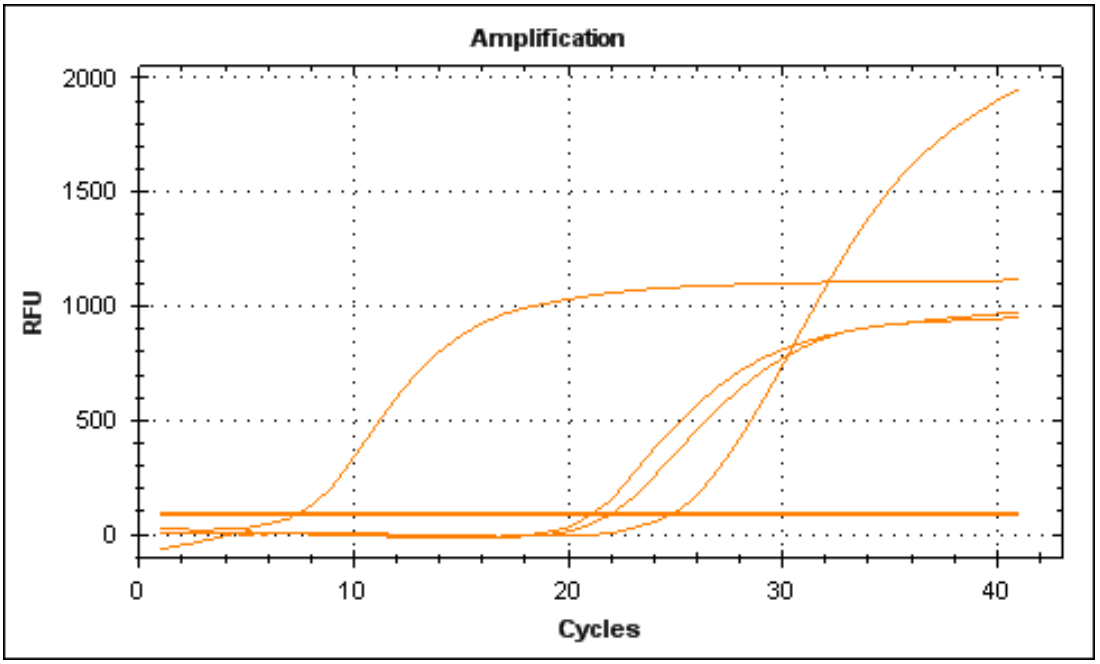

Figure 4. Results of Bacillus subtilis genome detection in the test material

Fluorescence accumulation, via the Rox channel, from cycle 21 onwards indicates the presence of Bacillus subtilis DNA in the sample. According to BLAST analysis, the amplification of this gene is only possible in bacteria belonging to the species Bacillus subtilis in 199 isolates/strains of the microorganism.

Thus, three of the eighteen isolates of endophytic bacteria, which were conditionally designated with the ciphers B. subtilis EFS3, B. subtilis EFS9 and B. subtilis EFS13, showed high antagonistic activity against the micromycete F. sporotrichioides, and the absence of pathogenic and toxic properties.

The presence of an overwhelming effect on phytopathogens in various strains of B. subtilis bacteria has been established in a large number of other studies, and the potential for using these microorganisms as biocontrol agents has been emphasized (Zhao et al., 2014; Pan et al., 2015; Chen et al., 2018). At the same time, B. subtilis species are heterogeneous both phenotypically and genotypically, therefore, the search for new strains can expand the number of practically important strains and more fully reveal the mechanisms of antagonistic interactions (Kopac et al., 2014; Li et al., 2020). Some researchers have observed suppression of virulence, changes in mycelial morphology, inhibition of normal conidia development, and destruction of the fungal cell wall when bacteria come into contact with microscopic fungi (Petatan-Sagahon et al., 2011). In our study, the isolates that showed the greatest antagonistic activity also had a broad enzymatic profile, which may indicate that there is a direct relationship between them. This conclusion was also made in other researchers. Thus, the

degradation of the cell wall of phytopathogenic micromycetes and the antimicrobial potential of antagonist bacteria were associated with the ability to produce hydrolases (Baysal et al., 2013; Wu et al., 2014; Wang et al., 2017), and it was also found that these enzymes can stimulate plant growth by promoting root colonization by bacteria (Zhao et al., 2014; Chen et al., 2019; Kushwaha et al., 2020).

CONCLUSION

High antifungal activity against the micromycete F. sporotrichioides, as well as the absence of pathogenic and toxic properties, was found in isolates of endophytic bacteria belonging to the species B. subtilis. The conducted studies allow us to conclude that the selected microorganisms can be used for introduction into the biocenosis and regulation of the density of the above-mentioned phytopathogenic microscopic fungus.

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REFERENCES

- Baysal O, Lai D, Xu H, Siragusa M, Çalışkan M, Carimi F, Teixeira JA and Tör M (2013) A proteomic approach provides new insights into the control of soil-borne plant pathogens by Bacillus species. PloS One 8(1): e53182.
- Beneduzi A, Ambrosini A and Passaglia L (2012) Plant growth-promoting rhizobacteria (PGPR): the potential as antagonists and biocontrol agents. Genetics and Molecular Biology 35: 1044-1051.
- Chen L, Heng J, Qin S and Bian K (2018) A comprehensive understanding of the biocontrol potential of Bacillus velezensis LM2303 against Fusarium head blight. PLoS One 13(6): e0198560.
- Chen L, Shi H, Heng J, Wang D and Bian K (2019) Antimicrobial, plant growth-promoting and genomic properties of the peanut endophyte Bacillus velezensis LDO2. Microbiological Research. 218:41-48.
- Choudhary, DK and Johri BN (2009) Interactions of Bacillus spp. and plants with special reference to induced systemic resistance (ISR). Microbiological Research 164: 493-513.
- Ekblad A, Wallander H, Godbold DL, Cruz C, Johnson D, Baldrian P and Kraigher H (2013) The production and turnover of extramatrical mycelium of ectomycorrhizal fungi in forest soils: role in carbon cycling. Plant and Soil 366(1-2): 1-27.

- Gindullin AI, Shamilova TA, Gindullina DA, Tremasov MY, Ivanov AV, Ivanov AA, Chernov AN, Mukminov MN and Shuralev EA (2015) Influence of probiotics spas and biosporin at T-2 toxication of broiler chickens. Research Journal of Pharmaceutical, Biological and Chemical Sciences 6(4): 2142-2150.
- Hallmann J and Berg G (2006) Spectrum and population dynamics of bacterial root endophytes. Microbial Root Endophytes. Part of the Soil Biology book series (SOILBIOL, vol. 9). Berlin, Heidelberg: Springer Berlin Heidelberg: 15-31.
- Hujslova M, Kubatova A, Bukovska P, Chudickova M and Kolarik M (2017) Extremely acidic soils are dominated by species-poor and highly specific fungal communities. Microb Ecol. 73(2): 321-337.
- Kadikov IR, Papunidi KK, Korchemkin AA, Vafin IF, Semenov EI, Idiatov II and Zakirova GS (2018) Comparative evaluation of agents for preventive therapy for combined intoxications with xenobiotics in poultry. Indian Veterinary Journal 95(7): 29-32.
- Kopac S, Wang Z, Wiedenbeck J, Sherry J, Wu M and Cohan FM (2014) Genomic heterogeneity and ecological speciation within one subspecies of Bacillus subtilis. Applied and Environmental Microbiology 80(16): 4842-4853.
- Kushwaha P, Kashyap PL, Srivastava AK and Tiwari RK (2020) Plant growth promoting and antifungal activity in endophytic Bacillus strains from pearl millet (Pennisetum glaucum). Brazilian Journal of Microbiology 51(1):229-241.
- Li Q, Liao S, Wei J, Xing D, Xiao Y and Yang Q (2020) Isolation of Bacillus subtilis strain SEM-2 from silkworm excrement and characterisation of its antagonistic effect against Fusarium spp. Canadian Journal of Microbiology. 66(6): 401-412.
- Mardanova AM, Hadieva GF, Lutfullin MT, Khilyas IV, Minnullina LF, Gilyazeva AG, Bogomolnaya LM and Sharipova MR (2016) Bacillus subtilis strains with antifungal activity against the phytopathogenic fungi. Agricultural Sciences 8(1): 1-20.
- Montealegre JR, Reyes R, Perez LM, Herrera R, Silva P and Besoain X (2003) Selection of bioantagonistic bacteria to be used in biological control of Risoctonia solani in tomato. Electronic Journal of Biotechnology 6(2): 115-127.
- Pan D, Mionetto A, Tiscornia S and Bettucci L (2015) Endophytic bacteria from wheat grain as biocontrol agents of Fusarium graminearum and deoxynivalenol production in wheat. Mycotoxin Res. 31(3): 137-143.

- Petatan-Sagahon I, Anducho-Reyes MA, Silva-Rojas HV, Arana-Cuenca A, Tellez-Jurado A, Cárdenas-Álvarez IO and Mercado-Flores Y (2011) Isolation of bacteria with antifungal activity against the phytopathogenic fungi Stenocarpella maydis and Stenocarpella macrospora. International Journal of Molecular Sciences 12(9): 5522-5537.
- Potekhina R, Semenov E, Medvedeva I, Osyanin K, Aleksandrova N and Shuralev E (2020) Comparative toxicity assessment of soil fungi isolated from Black Sea coasts. BioNanoSci. 10: 799-806.
- Sekomo C, Rousseau D, Saleh S and Lens P (2012) Heavy metal removal in duckweed and algae ponds as a polishing step for textile wastewater treatment. Ecological engineering. 44: 102-110.
- Qin S, Li J, Chen HH, Zhao GZ, Zhu WY, Jiang CL, Xu LH and Li WJ (2009) Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna, China. Applied and environmental microbiology 75(19): 6176-6186.
- Wang L, Wang N, Mi D, Luo Y and Guo J (2017) Poly-γ-glutamic acid productivity of Bacillus subtilis BsE1 has positive function in motility and biocontrol against Fusarium graminearum. Journal of Microbiology 55(7):554-560.
- Wu Y, Yuan J, Raza W, Shen Q and Huang Q (2014) Biocontrol traits and antagonistic potential of Bacillus amyloliquefaciens strain NJZJSB3 against Sclerotinia sclerotiorum, a causal agent of canola stem rot. Journal of Microbiology and Biotechnology. 24(10): 1327-1336.
- Zhang Z, Schwartz S, Wagner L and Miller W (2000) A greedy algorithm for aligning DNA sequences. Journal of Computational Biology 7(1-2): 203-214.
- Zhao Y, Selvara J.., Xing F, Zhou L, Wang Y, Song H, Tan , Sun, L, Sangare Folly YME and Liu Y (2014) Antagonistic action of Bacillus subtilis strain SG6 on Fusarium graminearum. PLoS ONE 9(3): e92486.
- Zheng Z, Wang YF, Bai H, Li Y. and Dong ZP (2019) First report of Fusarium incarnatum-equiseti species complex causing ear rot of foxtail millet in Northwest regions of China. New Disease Reports. 40.