SCREENING OF ENDOPHYTIC BACTERIA EXHIBITING ANTAGONISTIC ACTIVITY AGAINST FUSARIUM SPOROTRICHIOIDES MICROMYCETE

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Abstract. Within the framework of this study, the micromycete Fusarium sporotrichioides was isolated from wheat grain, a collection of isolates of endophytic microorganisms with antagonistic activity against this pathogen was formed, and their antagonistic activity was studied. By the mechanism of action, the isolates are classified as strains exhibiting fungistatic antibiotic antagonism, that is, inhibition of fungal growth occurred at a distance under the influence of antibiotic substances produced by the antagonist, with the formation of a “sterile” zone between cultures; and strains with fungistatic alimentary type of antagonism, expressed in stopping the growth of the pathogen upon contact with the antagonist colony. Stepwise screening of microorganisms-antagonists in in vitro and in vivo experiments made it possible to select technological and safe isolates with high antifungal activity and the possibility of using them as biofungicide producers. Three isolates from among the most active aboriginal endophytic microorganisms belonging to the species Bacillus subtilis are promising for introduction into the biocenosis with the aim of long-term regulation of the density of phytopathogenic populations of Fusarium sporotrichioides.

1. Introduction

Microscopic fungi contamination of food and feed raw materials from the moment of their procurement to processing, storage and sale is a significant problem in ensuring food security. At any stage of production, micromycetes, when favorable conditions arise, synthesize metabolites highly toxic to humans and animals, many of which have mutagenic, teratogenic, carcinogenic, immunosuppressive and cytotoxic properties [1,2,3]. Representatives of the genus Fusarium were detected from samples of feed and agricultural products taken in different regions of the Russian
Federation, which have a high toxigenic potential, which suggests a high risk of infection by fusariotoxins [4,5,6].

These micromycetes worsen the sowing quality of grain, inhibit the development of the root system and stem of plants, reduce the nutritional quality of grain and its processed products, and therefore are considered worldwide as one of the most harmful pathogens of agricultural crops [7,8,9,10].

Antagonist bacteria are highly promising and a safe alternative to chemical agents for combating phytopathogenic fungi as agents of biological protection of plants and feed, since they effectively inhibit the development and spread of pathogens, stimulate plant growth, and have a beneficial effect on microbiocenoses [11,12,13,14]. Isolation of new microorganisms exhibiting antagonistic properties is necessary to improve methods of biological control and containment of pathogens [15,16,17,18].

The purpose of our study was the screening of endophytic bacteria exhibiting antagonistic activity against Fusarium sporotrichioides, the causative agent of Fusarium with toxigenic properties.

2. Material and Methods
The object of the study was endophytic bacterial strains isolated from natural biotopes, cultivated on meat-peptone agar (NICF, Russia) in a thermostat at 37 °C, a field strain of micromycete Fusarium sporotrichioides, isolated from the surface of wheat seeds according to methodological. The antagonistic activity of the isolates was determined by the methods of counter cultures, agar blocks, modified streak, and delayed antagonism.

The fungistatic activity of the isolates was assessed by the degree of inhibition of the growth of the fungal mycelium and was calculated using the formula:

\[ I = \left(1 - \frac{A}{B}\right) \times 100\%, \]

where:
- \( I \) - the percentage of inhibition;
- \( A \) - the number of colonies in the experiment;
- \( B \) - the number of colonies in the control.

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

The condition for the applicability of microorganisms for the production of a biological product is the safety of the feed processed by them and the absence of pathogenicity of the isolates.

The identification of the isolates was carried out by morphological and cultural characters, the species was confirmed by the polymerase chain reaction method using gene-specific primers selected in the GenBank database.

Statistical processing of the obtained digital material was carried out by the method of variation statistics using the Microsoft Excel program.

3. Results and discussion
According to the mechanism of antifungal action, based on the evaluation of the results of setting up the experiment by the method of counter cultures, endophyte isolates were divided into those with a high rate of mobility and forming a sterile zone of antagonistic action. Of the isolates with mobility (Fig. 1), high inhibitory activity against the micromycete Fusarium sporotrichioides was manifested in EFS3, EFS10, EFS14, and EFS15; on day 10 of co-cultivation, they occupied the largest area of the nutrient substrate of the Petri dish, blocking further growth of the test culture of the fungus, near the growth zone, the mycelium of the pathogen was partially lysed. By the 30th day of incubation, the EFS5 isolate occupied half of the plate area; the test micromycete inhibited the development of the EFS2, EFS7, EFS12, and EFS16 isolates, growing over the area occupied by them.
On the 10th day of co-cultivation, the isolates formed a sterile zone with a width of 16.9 to 22.5 mm, however, on the 15th day, there was no sterile zone between the pathogen and isolates EFS6, EFS11 and EFS18, the zone of inhibition of the mycelium of the remaining isolates was 3.2-19.5 mm. By day 30, isolates EFS8, EFS9, EFS13, and EFS17 retained the sterile zone (Fig. 2).

The zone of inhibition of micromycete growth, established by the method of agar blocks, was observed during co-cultivation with 11 isolates out of 18 selected (Table 1). The largest zone was formed by the EFS9 culture - 27.00 ± 0.50 mm, the antagonistic activity of the isolate was the highest.
in comparison with the rest - 2.01. When the pathogen was incubated with EFS14, the retention zone of the fungal mycelium was 25.89 ± 0.41 mm, the activity was 1.81, EFS10 was 24.50 ± 0.35 mm and 1.81, EFS13 was 23.20 ± 0.35 mm and 1.72, EFS15 - 22.70 ± 0.35 mm and 1.68, EFS17 - 20.00 ± 0.50 and 1.48, EFS3 - 15.70 ± 0.35 and 1.16, EFS4 - 14.25 ± 0.19 and 1.06, respectively.

Table 1. Antagonistic activity of endophytes against Fusarium sporotrichioides, revealed by the method of agar blocks

| Isolate | Activity      |
|---------|--------------|
| EFS1    | 0.27±0.03    |
| EFS2    | 0            |
| EFS3    | 1.16±0.07    |
| EFS4    | 1.06±0.04    |
| EFS5    | 0            |
| EFS6    | 0            |
| EFS7    | 0.38±0.05    |
| EFS8    | 0.71±0.09    |
| EFS9    | 2.01±0.09    |
| EFS10   | 1.81±0.07    |
| EFS11   | 0            |
| EFS12   | 0            |
| EFS13   | 1.72±0.08    |
| EFS14   | 1.92±0.08    |
| EFS15   | 1.68±0.09    |
| EFS16   | 0            |
| EFS17   | 1.48±0.12    |
| EFS18   | 0            |

It was established by the modified stroke method that the isolated isolates to one degree or another had antagonistic activity against the test culture of the fungus, the zone of inhibition of the growth of the pathogen by the 7th day of co-cultivation varied from 3.6 to 15.5 mm (Table 2). The highest activity was shown by isolates EFS3, EFS10, EFS14 and EFS15, the zone of their growth retardation of the test fungus strain was, respectively, 14.17 ± 0.43mm, 13.17 ± 0.33mm, 14.50 ± 0.25mm and 15.17 ± 0.33mm.

Table 2. Antagonism of endophytes to Fusarium sporotrichioides, established by the dash method along the growth inhibition zone, mm

| Endophyte isolate | 2 days  | 4 days  | 7 days  |
|-------------------|---------|---------|---------|
| EFS1              | 4.35±0.35 | 4.10±0.20 | 3.98±0.21 |
| EFS2              | 7.70±0.35 | 7.50±0.35 | 7.33±0.17 |
| EFS3              | 15.10±0.35 | 14.33±0.47 | 14.17±0.43 |
| EFS4              | 5.00±0.00 | 5.10±0.25 | 5.17±0.33 |
| EFS5              | 4.89±0.21 | 5.33±0.37 | 5.21±0.19 |
| EFS6              | 5.00±0.00 | 5.20±0.25 | 5.17±0.21 |
| EFS7              | 5.00±0.20 | 5.00±0.00 | 4.83±0.17 |
The evaluation of the fungistatic action of the isolated isolates of native microorganisms was carried out by the method of delayed antagonism. When the pathogen was cultivated on a medium with inactivated cultures of bacteria, 4 isolates completely inhibited the growth of the fungus: EFS3, EFS9, EFS13, and EFS17. The activity of the remaining isolates was assessed by counting the number of grown micromycete colonies and expressed by the degree of inhibition of the pathogen growth (Fig. 3).

The degree of inhibition of fungal development by isolate EFS 14 in comparison with the most active strains was 12.2% lower, EFS15 - 16.7%, EFS10 - 21.1%. Isolates EFS7, EFS 8, EFS 12, and EFS 18 had minimal fungistatic activity, the degree of inhibition of the pathogen was 12.2 - 27.8%. The activity of the other isolates did not exceed 65%.

Thus, active isolates are detected using each of the methods, however, the completeness of the picture of the interaction between the antagonist and the pathogen is achieved by setting up several complementary experiments, which made it possible to establish the nature of the relationship
between the isolates and the pathogen, the degree of inhibition of mycelium growth, the width of the growth zone of the fungus in the presence of the antagonist, and the size of the area of diffusion of substances. From the block of isolate to the mycelium of the fungus with the formation of a sterile zone, morphological changes of cultures during the cultivation of the antagonist directly on the medium with the pathogen. Endophytes EFS3, EFS9, EFS13, and EFS17 had high antifungal potential during growth and activity of metabolites; therefore, they were selected for further research.

In addition to the established efficacy against Fusarium sporotrichioides and cultural characteristics, the enzymatic, pathogenic, and toxicity of the isolates were studied.

The assimilation of nutrients by microorganisms occurs under the influence of exoenzymes belonging to the class of hydrolases, most of them are inducible and are synthesized in response to the presence of an inducer substrate necessary for the cell in the medium. With an excess of nutrients and microorganisms in the environment, competition for nutrients becomes a factor in suppressing the germination of phytopathogen spores. With a small number of microorganisms and a lack of nutrients, the antagonistic properties of bacteria and their ability to lysis are manifested. The condition for effective lysis of pathogenic fungi and / or the use of fungal mycelium as a food source is the complex action of various hydrolytic enzymes.

As a result of the study, it was found that isolates of endophytic microorganisms with antagonism to Fusarium sporotrichioides had a high level of enzymatic activity (Table 3).

The presence of amylolytic activity in the isolates was evidenced by the staining of the nutrient medium on which they were cultivated, when Lugol's solution was added to a yellow-brown color. Strains EFS9 and EFS17 showed moderate lipolytic activity; insignificant acidification of the medium occurred during EFS3 cultivation. EFS3 isolate has cellulolytic activity. The rest of the strains did not decompose cellulose. The selected isolates exhibit catalase activity, as determined by the formation of a "foam cap". Isolates EFS3, EFS9, and EFS13 were able to produce chitinase.

| Isolate | Amylolytic | Proteolytic | Lipolytic | Celluloselytic | Catalase | Chitinase |
|---------|------------|-------------|-----------|----------------|----------|----------|
| EFS3    | +          | ±           | -         | +              | +        | +        |
| EFS9    | +          | +           | ±         | -              | +        | +        |
| EFS13   | +          | +           | ±         | -              | +        | +        |
| EFS17   | +          | +           | ±         | -              | +        | -        |

Note: "+" - pronounced activity; "±" - moderate or weakly expressed activity; "-" - lack of activity.

The results of the study of grain contaminated with Fusarium sporotrichioides and treated with the culture liquid of isolates are presented in Table 4. In the extract of samples of grain affected by the pathogen, the survival rate of ciliates representatives was 42.84%, the extract of endophyte EFS17 was 21.59% lower than the control, isolates EFS3, EFS9 and EFS13 7.60; 6.10 and 8.84%, respectively.

| Group    | Stylonychia mytilus survival,\% | Mice survival,\% |
|----------|---------------------------------|------------------|
| TF+EFS3  | 84,64±2,11                      | 100,00           |
| TF+EFS9  | 86,14±1,97                      | 100,00           |
| TF+EFS13 | 83,40±1,84                      | 100,00           |
In an experiment on white mice, it was found that grain contaminated with Fusarium sporotrichioides and treated with EFS17 isolate had toxic properties, the survival rate of animals when an aqueous solution of acetone extract was introduced was 80.00%. The rest of the endophytes had a detoxifying effect on the food and, when administered intraperitoneally, did not lead to the death of mice; when the animals killed by decapitation were dissected, no visible changes were observed in the internal organs.

The analysis of cultural, morphological, physiological and biochemical characteristics showed that the isolates are phenotypically different strains of the Bacillus subtilis species. The species was confirmed by polymerase chain reaction (PCR) using gene-specific primers selected in the GenBank database.

In an experiment on white rats and mice, the absence of visible changes in the clinical status and death of animals with intraperitoneal and oral administrations showed that the isolates lack virulent, toxic and toxigenic properties.

The results of the conducted studies allow us to conclude that the selected isolates of aboriginal endophytic microorganisms Bacillus subtilis EFS3, Bacillus subtilis EFS9, and Bacillus subtilis EFS13 possess high antagonistic activity against Fusarium sporotrichioides and do not exhibit pathogenic and toxic properties.

The manifestation of a suppressive effect on phytopathogens by various strains of Bacillus subtilis bacteria has been established in a large number of works, and their potential for use as biocontrol agents is emphasized [19,20,21,22]. It is known that B. subtilis species are heterogeneous both phenotypically and genotypically; therefore, the search and identification of new strains from different sources can expand the number of practically important strains and more fully reveal the mechanisms involved in antagonistic interactions [23,24,25,26]. A number of researchers noted significant changes in mycelium morphology caused by bacteria, destruction of the fungal cell wall and inhibition of the normal development of conidia, suppression of virulence [27,28,29,30]. The synthesized hydrolases make an important contribution to the antimicrobial potential of antagonists, since some hydrolytic enzymes are involved in the degradation of the cell wall of phytopathogenic micromycetes [31,32,33,34]. Pectinases and amylases promote bacterial root colonization and, therefore, can play an important role in stimulating plant growth [35,36]. In our study, the endophyte strains also showed broad enzymatic activity.

4. Conclusions

Three endophytic strains of bacteria belonging to the species Bacillus subtilis were selected, which showed high antifungal activity against the micromycete Fusarium sporotrichioides, the absence of pathogenic and toxic properties and, therefore, are promising for introduction into biocenosis with the aim of long-term regulation (biocontrolling) of the density of phytopathogenic populations.

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