Endophytic isolates of *Bacillus subtilis*: prospects of application for improving the quality of food raw materials

I I Idiyatov¹, A I Eroshin¹, S A Yusupov¹, E V Zdoroveva² and A M Tremasova¹

¹Federal Center for Toxicological, Radiation and Biological Safety, Nauchny Gorodok-2, Kazan, Russian Federation, 420075.
²Penza State Agrarian University, 30 Botanicheskaya St., Penza, 440014, Russia

E-mail: idiyatov_ilgiz@mail.ru

Abstract. Endophytic microorganisms are most closely in contact with agricultural plants, inhabit their internal tissues, provide protection from pathogens, and have a direct impact on plant health and productivity. This is the relevance of studying the properties of endophytic organisms for the regulation of population density of pathogenic micromycetes. In this work, a comparative assessment of the antifungal activity of endophytic isolates *B. subtilis* EFS3, *B. subtilis* EFS9, *B. subtilis* EFS13 was carried out with respect to field isolates of microscopic fungi of the genera *Fusarium*, *Aspergillus*, *Penicillium*, *Mucor*. The studied endophytic bacilli inhibited the growth of micromycetes both due to the antibiotic substances produced and by direct contact, i.e., antibiotic and alimentary types of antagonism were noted in the mechanism of antifungal action. Thus, the studied endophytic bacteria are able to influence the development of pathogenic fungi and can be used to develop biological products that contribute to improving the quality of food raw materials of plant origin.

1. Introduction

Pathogenic fungi cause a decrease in crop yields and the phytosanitary quality of the obtained plant raw materials and food products, pose a serious danger to human health, lead to significant economic losses in the agricultural and food industries [1-4].

The use of various agronomic techniques, breeding methods, chemical and biological preparations can reduce the impact of undesirable microflora on plants. However, the use of pesticides of chemical origin requires strict compliance with the necessary measures to prevent poisoning of people and animals, environmental pollution and food raw materials [5]. A safe and environmentally friendly alternative is the biological control of pathogenic organisms [6]. At the same time, endophytic microorganisms, mainly of the genus *Bacillus*, can provide reliable suppression of the vital activity of plant pathogens [1, 7, 8].

The aim of this study was to compare the antifungal activity of three endophytic isolates of *Bacillus subtilis*.

2. Materials and methods

The object of the study was endophytic isolates derived from the vegetative part of spring wheat of the Ester variety. The plants were collected during the earing phase, at the time of the greatest activity of the rhizosphere microbiota. The material was washed three times with distilled water, cut into
fragments, the ends of which were treated with paraffin, then sterilized \cite{9, 10}. After that, sterility control was carried out by placing part of the samples in nutrient media for the cultivation of bacteria and fungi and incubated under thermostat conditions under appropriate conditions. Having received a negative result, the second part of the samples was sterile milled, filled with sterile distilled water, then the suspensions were sown in Petri dishes on the surface of the FMH-agar (FBIS «State Research Center of Applied Microbiology and Biotechnology», Obolensk, Russian Federation) with subsequent cultivation at a temperature of 37 °C for 24 hours, pure cultures of isolates were obtained by replanting individual colonies.

Thus, 18 isolates were isolated, three of which, conventionally designated EFS3, EFS9 and EFS13, showed the highest antagonistic activity against the micromycete \textit{Fusarium sporotrichioides}, were characterized by broad enzymatic activity, lack of pathogenic properties \cite{11}.

An analysis of tintorial and physiological-biochemical signs, results of species identity confirmation by polymerase chain reaction using gene-specific primers and comparison of sequenced sequences of the 16SrDNA fragment with those available in the database of the NCBI international data bank (National Center for Biotechnological Information, USA) using the BLAST system (Basic Local Alignment Search Tool) showed that the isolates are phenotypically different strains of the species \textit{B. subtilis}. Field isolates of micromycetes \textit{Aspergillus flavus}, \textit{Aspergillus niger}, \textit{Fusarium sporotrichioides}, \textit{Fusarium oxysporum}, \textit{Penicillium griseofulvum}, \textit{Mucor} spp. were used in experiments. The cultivation of the microscopic fungi listed above was carried out on the agarized medium of Chapek (RPC «Biocompas-S», Russian Federation) under thermostat conditions at a temperature of 26 °C for 7-14 days.

The antagonistic activity of isolates to micromycetes was determined by the method of agar blocks and the method of counter cultures. The number of repetitions in each group was 5.

The method of agar blocks consisted in cultivating a bacterial isolate on a dense nutrient medium in a Petri dish until a «continuous lawn» was formed, followed by cutting out blocks from it with a sterile cork drill and transferring them to the surface of the agarized Chapek medium in another Petri dish previously seeded with a mushroom test culture. The test culture was seeded with a spatula, the agar blocks were applied with an upward growth at an equal distance from each other and from the edges of the cup, tightly pressing against the surface of the medium. The control was the cultivation of micromycetes without an antagonist. The cups were incubated in a thermostat at a temperature of 26 °C. The account of the experience was carried out on the 2nd, 4th and 7th days. After 7 days of cultivation, the diameter of the mycelium growth suppression zone was measured in two mutually perpendicular directions. The activity of antagonist bacteria \textit{A} was calculated by the formula:

$$A = \frac{D}{d},$$

where \(D\) – is the diameter of the growth retardation zone of the mycelium of the fungus, mm; \(d\) – the diameter of the culture application site, 13 mm.

When setting up the experiment by the method of double (counter) cultures, the isolates of micromycetes and antagonist were grown separately on agarized media under appropriate conditions. Then, a block with the mycelium of the fungus was cut out with a sterilized drill and placed on the surface of the medium in a Petri dish, at a distance of 60 mm from it, the culture of the antagonist was applied with a stroke.

Cups with a micromycete without an isolate served as a control. The cups were incubated in a thermostat at 26 °C, the experience was taken into account on the 5th, 10th, 15th and 20th days of cultivation, noting the growth of cultures, the nature of their interaction, the zone of inhibition of the micromycete \cite{12}. The degree of inhibition of mycelium growth of the pathogen \textit{Inhibition}, %, was determined by the formula \cite{13}:

$$\text{Inhibition} = \left\{ \frac{(R - r)}{R} \times 100 \right\},$$

where \(R\) – radial growth of the fungus in control; \(r\) – radial growth of the fungus in the presence of bacteria.
Statistical processing of the results was carried out using the Microsoft Excel 2010 program. To compare the data, a two-sample Student t-test was used, the critical level of statistical significance ($p$) was assumed to be 0.05.

3. Results
The endophytic bacteria studied by us during co-cultivation with micromycetes had an effect on their morphology, character, growth intensity, and showed antagonistic activity against all tested micromycetes (Table 1). The diameter of the growth retardation zone of $A. flavus$ and $A. niger$ around the block with $B. subtilis$ EFS13 isolate was, respectively, 19.00±1.05 and 19.25±0.99 mm, the activity of the isolate was 1.46 and 1.48. The activity of $B. subtilis$ EFS3 in relation to these microscopic fungi was lower in comparison with EFS13, respectively, by 15.75 and 15.54 %, in $B. subtilis$ EFS9 isolate – by 18.49 and 22.97 %. The site of inhibition of $F. sporotrichioides$ growth by EFS3 isolate was 17.50±0.75 mm, the activity of EFS13 isolate was 8.15 % higher, EFS9 – by 28.89%. In relation to $F. oxysporum$, the greatest activity was demonstrated by its $B. subtilis$ EFS3 isolate (17.25±0.55 mm), the inhibitory effect of EFS13 and EFS9 was lower by 3.00 and 12.03 %, respectively. The values of the activity of the studied bacteria in relation to $P. griseofulvum$ did not have significant differences.

The diameter of the growth retardation zone of the $Mucor$ spp. micromycete around the EFS3 isolate block was the smallest and amounted to 14.25±0.73 mm, its activity was 1.10, EFS9 activity was 20.91 % higher, EFS13 – 26.36 %.

| Test culture     | $B. subtilis$ EFS3 | $B. subtilis$ EFS9 | $B. subtilis$ EFS13 |
|------------------|--------------------|--------------------|--------------------|
| $A. flavus$      | 1.23±0.10          | 1.19±0.09          | 1.46±0.08          |
| $A. niger$       | 1.25±0.07          | 1.14±0.08          | 1.48±0.08          |
| $F. sporotrichioides$ | 1.35±0.06       | 1.74±0.06          | 1.46±0.07          |
| $F. oxysporum$   | 1.33±0.04          | 1.17±0.06          | 1.29±0.11          |
| $P. griseofulvum$| 1.21±0.07          | 1.21±0.04          | 1.17±0.02          |
| $Mucor$ spp.     | 1.10±0.06          | 1.33±0.09          | 1.39±0.08          |

Thus, the studied $B. subtilis$ isolates, when co-cultured with test strains of micromycetes, not only preserved the place of application, but also formed a zone of growth retardation of microscopic fungi outside it, which indicates the presence of antifungal activity in bacteria, the mechanism of which we will try to reveal in experiments using the method of double (counter) cultures and the detection of metabolic activity.

Thus, $B. subtilis$ EFS3 had the highest growth intensity, competing with test cultures of fungi for nutrients, occupying the largest area of the nutrient substrate and blocking further growth of micromycetes (Table 2). The formation of a sterile zone of antagonistic action was noted between the colonies of microscopic fungi and the isolate of $B. subtilis$ EFS9 and $B. subtilis$ EFS13, which should be associated with the production of fungistatic exometabolites (Table 3).

The ability of representatives of this type of microorganisms to secrete antifungal compounds has also been shown by other researchers [12, 13].

$B. subtilis$ EFS13 had the greatest inhibitory effect on the growth of test cultures of micromycetes (Figure 1). The isolate suppressed the development of $A. flavus$ and $A. niger$ fungi by 54.73 and 56.39 %, respectively, the activity of the EFS3 isolate in comparison with it was lower by 5.63 and 8.53 %, and EFS9 – by 6.04 and 9.00 %. The vital activity of microscopic fungi $F. sporotrichioides$ and $F. oxysporum$ with $B. subtilis$ EFS13 isolate was suppressed by 56.61 and 53.22 %, the efficiency of EFS3 and EFS9 endophytes was slightly lower.

Inhibitory ability of isolates EFS13 and EFS9 against $P. griseofulvum$ and $Mucor$ spp. it was, respectively, about 50 and 55 %, the EFS3 isolate was less active, the value of the analyzed indicator was 45.21 and 38.31 %.
Table 2. Antagonistic activity of B. subtilis EFS3, manifested in an elementary type of antagonism against test micromycetes (mycelium growth from the seed block, mm).

| Group          | Cultivation period, day |
|----------------|-------------------------|
|                | 5          | 10       | 15       | 20       |
|                | A. flavus | A. niger | F. sporotrichioides | F. oxysporum | P. griseofulvum | Mucor spp. |
| Experiment     | 23.30±1.05a | 25.60±0.84a | 27.60±1.64a | 29.20±2.56a |
| Control        | 30.50±1.00 | 43.20±1.08 | 53.20±2.07 | 64.10±2.06 |
| Experiment     | 24.20±1.56a | 24.50±0.71a | 26.90±0.87a | 29.70±1.27a |
| Control        | 31.70±1.17 | 40.40±1.15 | 50.10±2.12 | 63.30±2.01 |
| Experiment     | 20.10±1.28a | 21.20±1.24a | 24.50±1.39a | 26.00±2.09a |
| Control        | 31.10±1.23 | 40.00±1.12 | 47.80±1.52 | 67.00±1.66 |
| Experiment     | 21.00±1.00a | 23.30±0.89a | 24.90±1.12a | 25.50±1.79a |
| Control        | 30.30±1.11 | 40.60±1.20 | 49.50±0.97 | 61.20±2.10 |
| Experiment     | 23.20±1.29a | 25.60±0.91a | 27.20±1.78a | 29.80±1.52a |
| Control        | 31.50±1.58 | 42.60±1.52 | 48.20±1.64 | 60.20±1.98 |
| Experiment     | 33.00±1.27a | 35.30±1.76a | 38.90±1.12a | 40.50±1.75a |
| Control        | 33.00±1.27 | 57.30±2.07 | 68.10±1.66 | 69.60±0.45 |

*a – p <0.05, the comparison was carried out with the «control» group.

Table 3. Antagonistic activity of B. subtilis isolates, manifested in the antibiotic type of antagonism against test micromycetes («sterile» inhibition zone, mm).

| Test culture | Cultivation period, day |
|--------------|-------------------------|
|              | 10       | 15       | 20       |
| B. subtilis EFS9 |                |          |          |
| A. flavus   | 15.30±0.58 | 14.20±0.65 | 13.50±0.56 |
| A. niger    | 15.00±0.79 | 13.60±0.67 | 13.00±0.94 |
| F. sporotrichioides | 18.20±0.65 | 18.80±0.82 | 17.30±0.93 |
| F. oxysporum | 16.20±0.55 | 16.00±0.79 | 15.20±0.74 |
| P. griseofulvum | 17.40±0.97 | 16.80±0.82 | 16.10±0.80 |
| Mucor spp. | 16.10±0.57 | 15.20±0.65 | 15.00±0.94 |
| B. subtilis EFS13 |                |          |          |
| A. flavus   | 19.60±0.84 | 18.60±1.04 | 17.80±1.14 |
| A. niger    | 22.50±1.12 | 20.20±1.19 | 19.30±1.08 |
| F. sporotrichioides | 21.20±0.96 | 19.00±1.00 | 17.90±0.76 |
| F. oxysporum | 19.60±0.91 | 18.90±1.15 | 18.10±1.01 |
| P. griseofulvum | 17.60±1.04 | 17.20±0.89 | 16.50±1.00 |
| Mucor spp. | 18.00±0.79 | 16.80±0.96 | 16.10±0.97 |

Figure 1. The degree of inhibition of the development of microscopic fungi by endophytic isolates of B. subtilis (on day 20 of the study), %.
4. Discussion
The mechanisms involved in biological control are diverse. Identification of the relationship between antagonists and toxigenic fungi is a prerequisite for the development of effective means of biological control [14]. According to the results of our studies, endophytic isolates of *B. subtilis* inhibited the development of the studied micromycetes, including through the production of antifungal compounds. This fact was also established by other scientists, since lipopeptides (iturin, fengicin, surfactin), polypeptides, lytic enzymes, pyoverdines or bacteriocins, ammonia, potassium cyanide, siderophores, salicylic acid, volatile organic compounds secreted by bacteria of this species showed activity against various types of microscopic fungi [15-21]. The mechanism of action of these substances was explained by osmotic processes, increased membrane permeability, solubilization and subsequent destruction of the fungal cell wall [14, 19]. Radovanović et al. an antifungal effect was established in the *Bacillus* spp. strain against micromycetes *Aspergillus flavus*, *Fusarium graminearum*, *Mucor* spp. and *Alternaria* spp., which was explained by the action of highly stable secondary metabolites: iturin, bacillomycin D, mycosubtilin and surfactin [1]. In addition, when exposed to antagonist bacilli, noticeable morphological changes in the structure of the mycelium of micromycetes were noted, which were characterized by an increase and swelling of hyphae, extensive vacuolization, and the presence of cells devoid of cytoplasm [1, 7, 14, 22]. Thus, *B. subtilis* bacteria have the potential to be used as biocontrol agents. Of course, the antagonistic potential of microorganisms varies significantly and depends on phenotypic and genotypic factors, the search for new effective isolates allows us to more fully reveal the mechanisms involved in antagonistic interactions, contributes to an increase in the arsenal of antifungal agents [23].

5. Conclusions
According to the research results, it was found that isolates of endophytic bacteria *B. subtilis* (EFS3, EFS9, EFS13) have antifungal action against field isolates of microscopic fungi of the genera *Fusarium*, *Aspergillus*, *Penicillium*, *Mucor*, in the mechanism of which antibiotic and alimentary types of antagonism were revealed. The different degree of inhibition of the development of micromycetes is explained by the phenotypic and genetic features of both the fungi themselves and the antagonist bacteria. Thus, *B. subtilis* isolates (EFS3, EFS9, EFS13) can be used for the development of biological products that contribute to improving the quality of food raw materials of plant origin.

Acknowledgments
The work was carried out with the support of the Grants Council of the President of the Russian Federation as part of the implementation of grant MK-1582.2020.11 (Agreement No. 075-15-2020-225 dated 04/27/2020).

References
[1] Radovanović N, Milutinović M, Mihajlovski K, Jović J, Nastasijević B, Rajilić-Stojanović M and Dimitrijević-Branković S 2018 Biocontrol and plant stimulating potential of novel strain *Bacillus* spp. PPM3 isolated from marine sediment *Microbial Pathogenesis* **120** 71–8
[2] Zalila-Kolsi I, Mahmoud A B, Ali H, Sellami S, Nasfi Z, Tounsi S and Jamoussi K 2016 Antagonist effects of *Bacillus* spp. strains against *Fusarium graminearum* for protection of durum wheat (*Triticum turgidum* L. subsp. durum) *Microbiological Research* **192** 148–58
[3] Tarasova E Y, Matrosova L E, Tanaseva S A, Mishina N N, Potekhina R M, Ermolaeva O K, Smolentsev S Y, Tremasova A M, Kadikov I R, Egorov V I, Aslanov R M and Semenov E I 2020 Protective effect of adsorbent complex on morphofunctional state of liver during chicken polycystic disease *Systematic Reviews in Pharmacy* **11** 264–8
[4] Idiyatov I I, Valiullin L R, Birulya V V, Shangaraev N G, Ragain I S, Tremasov M I, Lekishvili M V and Nikitin A I 2017 Cytotoxic activity of T-2 toxin for a immortalized of cattle fetal lung epithelium cells *Genes & Cells* **12(1)** 41–6
[5] Masiello M, Somma S, Ghionna V, Logrieco A F and Moretti A 2019 In vitro and in field response of different fungicides against *Aspergillus flavus* and *Fusarium* species causing ear rot disease of maize *Toxins* **11(1)** 11
[6] Fira D, Dimkić I, Berić T, Lozo J and Stanković S 2018 Biological control of plant pathogens by Bacillus species. *Journal of Biotechnology* **285** 44–55

[7] 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. *Mycotaxon Res.* **31** 137–43

[8] Khabirova S R, Idiyatov I I, Shuralev E A and Tremasova A M 2021 Metabolite-associated enzymatic properties of microorganisms with an antagonistic effect on *Aspergillus* and *Fusarium* fungi, pathogenic for crops and farm animals. *IOP Conf. Ser. Earth Environ. Sci.* **723** 022022

[9] Hallmann J and Berg G 2006 Spectrum and population dynamics of bacterial root endophytes. *Microb. Root Endophytes* **9** 15–31

[10] Qin S, Li J, Chen H H, Zhao G Z, Zhu W Y, Jiang C L, Xu L H and Li W J 2009 Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna, China. *Appl Environ Microbiol.* **75**(19) 6176–86

[11] Idiyatov I I, Tremasova A M, Tremasov Y M, Valiullin L R, Kalimitchenko V P, Rud V Y and Semenov A M 2021 Screening of endophytic bacteria exhibiting antagonistic activity against *Fusarium sporotrichioides* microcyst. *IOP Conf. Ser.: Earth and Environmental Science* **663** 012047

[12] Montealegre J R, Reyes R, Perez L M, Herrera R, Silva P and Besoaín X 2003 Selection of bioantagonistic bacteria to be used in biological control of *Rhiocetonia solani* in tomato. *Electronic Journal of Biotechnology* **6**(2) 116–27

[13] Mardanova A M, Hadieva G F, Lutfullin M T, Khilyas I V, Minnullina L F, Gilyazeva A G, Bogomolnaya L M and Sharipova M R 2017 *Bacillus subtilis* strains with antifungal activity against the phytopathogenic fungi *Agricultural Sciences* **8** 1–20

[14] Zhao Y, Selvaraj J N, Xing F, Zhou L, Wang Y, Song H, Tan X, Sun L, Sangare L, Folly Y M E and Liu Y 2014 Antagonistic action of *Bacillus subtilis* strain SG6 on *Fusarium graminearum* PLoS ONE **9**(3) e92486

[15] Waewthongrak W, Pisuchpen S and Leelasuphakul W 2015 Effect of *Bacillus subtilis* and chitosan applications on green mold (Penicillium digitatum Sacc.) decay in citrus fruit. *Postharvest Biology and Technology* **99** 44–9

[16] Brzezinska M S, Kalwasińska A, Świątczak J, Żero K and Jankiewicz U 2020 Exploring the properties of chitinolytic *Bacillus* isolates for the pathogens biological control. *Microbial Pathogenesis* **148** 104462

[17] Santoro M, Cappellari L, Giordano W and Banchio E 2015 Production of volatile organic compounds in PGPR *Handbook for Azospirillum* 307–17

[18] Senol M, Nadaroglu H, Dikbas N, and Kotan R 2014 Purification of chitinase enzymes from *Bacillus subtilis* bacteria TV-125, investigation of kinetic properties and antifungal activity against *Fusarium culmorum* *Ann Clin Microbiol Antimicrob*. **13** 35

[19] Kesaulya H, Hasinu J V and Tuhumury G N C 2018 Potential of *Bacillus* spp. produces siderophores insuppressing the wilt disease of banana plants *IOP Conf. Ser. Earth Environ. Sci.* **102** 012016

[20] Zheng M, Shi J, Shi J, Wang Q and Li Y 2013 Antimicrobial effects of volatiles produced by two antagonistic *Bacillus* strains on the anthracnose pathogen in postharvest mangos *Biol. Control* **65** 200–206

[21] Beneduzi A, Ambrosini A and Passaglia L M 2012 Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents *Genet. Mol. Biol.* **35**(4) 1044–51

[22] Dihazi A, Jaiti F, Taktak W, kilani-Feki O, Jaoui S, Driouich A, Baaziz M, Daayf F and Serghini M A 2012 Use of two bacteria for biological control of bayoud disease caused by *Fusarium oxysporum* in date palm (Phoenix dactylifera L) seedlings. *Plant Physiology and Biochemistry* **55** 7–15

[23] Kopac S, Wang Z, Wiedenbeck J, Sherry J, Wu M and Cohan F M 2014 Genomic heterogeneity and ecological speciation within one subspecies of *Bacillus subtilis* *Appl. Environ. Microbiol.* **80**(16) 4842–53