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ISOLATION AND CHARACTERIZATION OF ANTIFUNGAL METABOLITES OF *Bacillus subtilis* STRAINS BZR 336G AND BZR 517 USING THE MODIFIED BIOAUTHOGRAPHY METHOD

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Abstract

The ability of *B. subtilis* to produce a large number of biologically active metabolites of diverse structure and properties can largely determine its fungicidal effect against particularly dangerous pathogenic fungi. In this regard, we have studied the fungicidal activity and properties of the antifungal metabolites of active strains *B. subtilis* BZR 336g and BZR 517. The approach we used, including purification by extraction with ethyl acetate, thin-layer chromatography (TLC), UV fluorescence, tests with detection reagents, spectroscopy and bioautography, showed the potential of the studied *B. subtilis* strains to accumulate the complex of active metabolites exhibiting antifungal effect from fungistatic to fungicidal. Bioautographic method was modified by use of the causative agents of harmful diseases, *F. oxysporum* var. *orthoceras* and *Alternaria* sp. as test organisms, which allowed us to assess in vitro the antifungal activity. Patterns of the antifungal compounds differed between the strains in TLC mobility and UV fluorescence. The impact on fungal growth also differed, from slight changes in mycelium growth to total suppression. In strain *B. subtilis* BZR 336g the most pronounced inhibition of *F. oksysporum* var. *orthoceras* BZRP1 growth was in bands with Rf 0.39 and 0.96. Significant *Alternaria* sp. BZRP8 growth inhibition was also observed in two bands, with Rf 0.42 and 0.96. *B. subtilis* BZR 517 synthesizes metabolites exhibiting fungitoxic properties in four bands with Rf 0.42, 0.84. 0.92 and 0.96 against *F. oxysporum* var. *orthoceras* BZRP1, and in two bands with Rf 0.42 and 0.96 against *Alternaria* sp. BZRP8. It is known that, in addition to the direct action, surfactins and fengicins of *B. subtilis* prevent the adhesion of competitive microbes on the plant. In out tests, spraying TLC plates with distilled water revealed bands with hydrophobic properties (non-wetting white zones), which can enhance the antagonistic effect of *B. subtilis.* Test with detection reagents visualized the presence of aromatic amines, phenols, phenolic steroids, methyl hydrazines, that is, compounds that probably play a certain role in the bioactivity manifestation. The weak staining of the metabolites with ninhydrin, as well as the absence of characteristic precipitation during acidification of the culture medium or adding ammonium sulfate, may indicate the presence of only minor amounts of amino acids and peptides. Spectrophotometrically we detected absorption peaks at 205-217 nm wavelengths, which indirectly evidences the presence of polyene structures. Our data allow us to hypothesize mechanisms underlying antifungal effects in the studied *B. subtilis* strains. These effects may be due to hydrolysis of phenolic compounds, since the detecting reagents indicate various phenolic derivatives, and, according to many researchers' opinion, active strains, when interacting with a phytopathogen, produce hydrolytic enzymes. As a result, the active groups of phenols can be released, which enhances the antifungal activity of the synthesized metabolites via effect on both fungal cell walls and other cell structures.

Keywords: *Bacillus subtilis*, microbiological preparations, antifungal metabolites, fungitoxicity, thin-layer chromatography, bioautography, detection reagents, phenolic compounds, polyene structures

Hay bacillus (*Bacillus subtilis*) produces various antimicrobial metabolites: lipopeptides, polypeptides, enzymes, non-peptide compounds [1-3], which

largely determines its fungicidal effect against particularly dangerous pathogenic fungi. The structure and mechanism of action of lipopeptide fungicides, which include active peptides from the families of iturins, surfactins, and fengycins [4, 5], are studied in most detail. It has been proven that the synthesis of lipopeptides of *B. subtilis* plays a key role in the suppression of pathogens in natural conditions, and the production of iturins and fengycins is determined by the presence of phytopathogens in the environment. High efficacy against filamentous fungi is associated with the ability of *B. subtilis* metabolites to influence membranes through interaction with ergosterol, with the formation of pores and subsequent release of monovalent cations from cells which are therefore lysed [5, 6]. For lipopeptides from different families, the specific pore formation mechanisms are different [7]. As a rule, bacteria strains with a high content of lipopeptide antibiotics have a higher antagonistic activity and a wide spectrum of action.

On the other hand, plant polysaccharides stimulate the formation of surfactin produced in the first hours of the interaction of bacilli with root tissues [8, 9]. The fungicidal activity is also associated with the presence of surfactants in bacilli, which are amphipathic molecules with polar and hydrophobic regions [10]. Surfactins are among the most effective biosurfactants (surfactants of biological origin). Having a structure similar to iturins and antagonistic properties, surfactin molecules, in contrast to iturins, contain amino acids with hydrophobic radicals and β-hydroxylated fatty acid [11]. In addition to direct action, surfactins and fengycins of *B. subtilis* prevent the adhesion of competitive microbes and can induce systemic resistance to pathogens and unfavorable abiotic factors in plants [12). Interestingly, lipopeptide antibiotics can be perceived by plant cells as a signal to initiate defense mechanisms, that is, to be elicitors [13]. Surfactins and fengycins, as elicitors of the host plant immune response, have been shown to significantly induce the defensive response of beans to the causative agent of gray mold *Botrytis cinerea* [14].

In this research, metabolites that suppress the development of harmful phytopathogenic fungi were first detected in the *Bacillus subtilis* BZR 336g and BZR 517 strains. Using the original analytical methods and approaches, we determined the properties and chemical structure of the identified compounds. The results obtained give reason to consider both of the studied strains as potential producers of biofungicides.

The aim of this research was to isolate, study the chemical structure and quantify the antifungal activity of metabolites released into the culture fluid by two promising strains of *B. subtilis*.

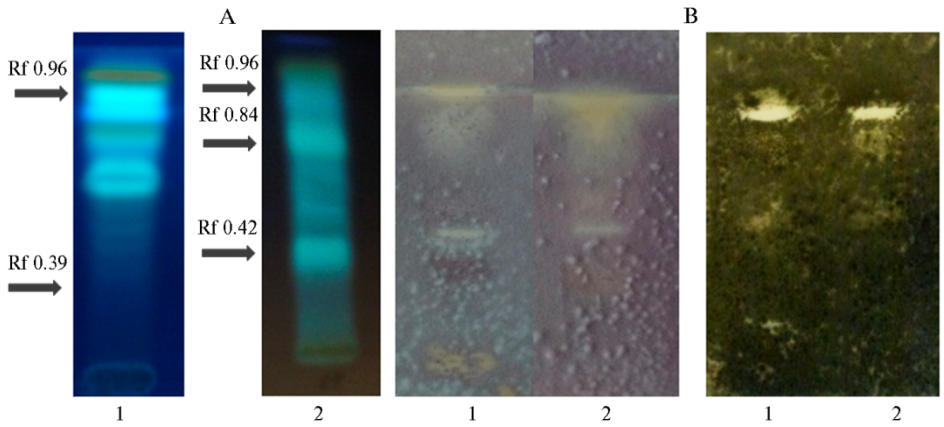
Techniques. We used *B. subtilis* BZR 336g and BZR 517, the producers of experimental biologicals [15-17], as well as cultures of phytopathogenic fungi *Fusarium oxysporum* var. *orthoceras* BZRP1 (a causative agent of root rot in grain and oil crops) and *Alternaria* sp. BZRP8 (a causative agent of Alternaria blight in fruit crops) from the collections of the All-Russian Research Institute of Biological Plant Protection (ARRIBPP).

The liquid culture of biofungicide based on bacterial strains was obtained by the method of periodic culture (New Brunswick Scientific thermostating systems, Excella E25, USA) for 48 h at 180 rpm. The composition of the nutrient medium and the culture conditions are commercial secrets of ARRIBPP (Order No. 42-p dated November 28, 2012). After the culturing was completed, the liquid culture of *B. subtilis* was centrifuged for 20 min at 12,000 g, the supernatant was extracted for 1 h with three volumes of ethyl acetate. The ethyl acetate extract was evaporated, the dry residue was dissolved in a minimum amount of ethyl acetate and analyzed by ascending thin-layer chromatography (TLC) on

20×20 cm plates with modified Kieselgel 60 silica gel (Merck, Germany), layer thickness 2 mm, solvent system ethyl acetate:ethanol:water (40:15:15). After viewing under UV light at λ = 366 nm and removing traces of solvents, the TLC-plates were soaked in the potato-glucose nutrient medium, then in a suspension of propagules of the test fungus and placed for 48-50 h in a moist chamber at 24 °C for *Alternaria* sp. BZRP8 and 28 °C for *F. oxysporum* var. *orthoceras* BZRP1. The localization of the active components was detected by the formation of zones of absence or inhibition of the fungus growth [18, 19].

For further study of the active components of the culture fluid, the required amount of ethyl acetate extract was separated by TLC as described above. The zones corresponding to the active antifungal compounds were scraped off and eluted with ethyl acetate. For qualitative analysis of eluates, specific detection reagents were used [19]. The absorption spectra of the active fractions of *B. subtilis* BZR 336g and BZR 517 metabolites were determined at λ = 200-400 nm using a SmartSpec Plus spectrophotometer (Bio-Rad, USA).

Results. When viewing the TLC-plates under UV_{366} light, we found a significant set of produced metabolites which differed in chromatographic mobility and character of fluorescence (Fig., A) in both strains of *B. subtilis*. At the same time, the observed patterns of the strains differed.



The results of thin-layer chromatography (A, fluorescence in UV_{366} light) **and bioautograms** (B, on the left with the *Fusarium oxysporum* var. *orthoceras* BZRP1 test culture, on the right with *Alternaria* sp. BZRP8) **of ethyl acetate extracts isolated from culture liquids of *Bacillus subtilis* BZR 336g** (1) **and BZR 517** (2) **strains** (plates with modified Kieselgel 60 silica gel, Merck, Germany; after chromatographic separation of metabolites, silica gel was soaked in the potato-glucose nutrient medium, then in a suspension of propagules of the fungus and placed for 48-50 h in a moist chamber).

To identify the active fractions of metabolites by the method of bioautography, phytopathogenic fungi *F. oxysporum* var. *orthoceras* BZRP1 and *Alternaria* sp. BZRP8 were used as test objects to evaluate the antifungal effect against causative agents of root rot in grain crops and Alternaria blight in fruit crops. Both studied strains synthesized a complex of active compounds with fungistatic and fungicidal properties (see Table). Four most pronounced zones of complete growth inhibition of the fungus *F. oxysporum* var. *orthoceras* BZRP1 were found for BZR 517 strain, and only two for BZR 336g. The character of the fungus growth also differed from the usual due to the fungistatic effect of metabolite compounds (see Fig., B). The number of metabolite zones with fungitoxic effects significantly reduced on bioautograms with the *Alternaria* sp. BZRP8 fungus: only two most distinct zones of inhibition of the fungus were identified in both strains (see Table). However, in BZR 517, the manifestation of antifungal properties was more pronounced than in BZR 366g (see Fig., B).

Mobility, UV_{366}-fluorescence and antifungal properties of ethyl acetate extracts from culture fluid produced by *Bacillus subtilis* strains

Strain	Rf	Fluorescence	Antifungal activity	
			Fusarium oxysporum var. *Orthoceras* BZRP1	*Alternaria* sp. BZRP8
B. subtilis BZR 336g	0.32	Weak gray	–	–
	0.39	Bluish-gray	+++	–
	0.42	Bluish-gray	+	++
	0.51	Gray-blue	–	–
	0.57	Gray	–	–
	0.73	Bright whitish	+	–
	0.78	Bright greenish	+	–
	0.81	Dark blue	+	–
	0.84	Light greenish	-	–
	0.87	Dark green	+	–
	0.91	Bright whitish	+	–
	0.94	Bright whitish	+	+
	0.96	Intense dark green	+++	+++
B. subtilis BZR 517	0.32	Gray	–	–
	0.39	Bright light blue	+	–
	0.42	Gray	+++	++
	0.48	Light blue	+	–
	0.56	Light blue	+	–
	0.67	Gray	+	–
	0.75	Bright light blue	+	–
	0.84	Gray	++	–
	0.92	Light blue	+++	+
	0.96	Greenish yellow	+++	+++

Note. "+++" means fungicidal activity, "++" means average fungistatic activity, "+" means weak fungistatic activity; dashes indicate the absence of growth inhibition (separation of components by thin-layer chromatography with modified Kieselgel 60 silica gel, Merck, Germany).

Spraying the plate with distilled water allowed us to identify components with hydrophobic properties (manifested as white spots), one zone with Rf 0.84 in BZR 517 strain, two zones with Rf 0.75 and 0.84 in BZR 336g. According to many authors, the presence of surface-active metabolites can enhance the antagonistic properties of bacteria due to the influence on their ability to quickly spread over the leaf surface and in the rhizosphere [8].

With detecting reagents, we found functional groups that can contribute to the appearance of the biological activity of metabolites in the strains studied. When sprayed with a freshly prepared mixture of 0.1 M $FeCl_3$ and 0.1 M potassium ferricyanide (1:1), one zone of a BZR 517 metabolite (with Rf 0.92) and three zones of BZR 336g metabolites (with Rf 0.42, 0.57 and 0.73) were stained in a bright dark blue color, which indicates the presence of active compounds of aromatic amines, phenols and phenolic steroids. Navy blue staining of zones with a solution of Na_2CO_3 and Folin's reagent in BZR 517 and BZR 336g strains probably indicates the presence of methyl hydrazines and phenolcarboxylic acids. Using a 0.25% ninhydrin solution, the presence of free amino groups is detected in the same zone with Rf 0.73 for BZR 336g strain (a yellow-orange spot). Two zones with Rf 0.42 and 0.73 in BZR 336g stained in dark red with 0.5% "fast red B" salt solution may indicate the presence of phenols and hydroxybenzophenones [20].

According to the study, some assumptions can be made regarding the nature of the action of antifungal metabolites in the *B. subtilis* BZR 336g and BZR 517 strains. Since the detecting reagents indicate the presence of various phenolic derivatives, and the active strains, when interacting with the phytopathogen, produce hydrolytic enzymes [21, 22], then one of the mechanisms by which the phytopathogenic microorganisms are suppressed can be the hydrolysis of phenolic compounds. At the same time, the active groups of phenols are released, which contributes to the enhancement of the antifungal activity of the

synthesized metabolites. Since the attempt to precipitate the studied metabolites by acidification or ammonium sulfate was not successful, we assume that most of them have a non-peptide structure. There is evidence that some strains of *B. subtilis* form polyene antibiotics with conjugated double bonds that can inhibit the growth of phytopathogenic fungi [23]. A spectrophotometric study of the active fractions of metabolites in *B. subtilis* BZR 336g and BZR 517 showed that both strains produced compounds with an absorption maximum at λ = 205-217 nm, which serves as indirect evidence of the presence of short polyene chains in the structure of the studied substances. For long polyene chains, the absorption bands fall into the visible region, that is, the compound becomes colored (Rf 0.96) [24].

Many authors have shown that enhanced production of active metabolites contributes to an increase in the antagonistic activity of *B. subtilis* strains selected as promising biofungicidal agents. In cotton disease caused by *Rhizoctonia solani*, the antifungal effect of the *B. subtilis* NCD-2 strain on the pathogen was found to be mainly related to fengycin lipopeptides [25]. On melon leaves, lipopeptides from the families of iturins and fengycins [26] played a key role in the antagonism of four strains of *B. subtilis* against the pathogen *Podophaera fusca*. Fengycins synthesized by *B. subtilis* were of primary importance in the protection of leguminous plants from *Pythium ultimum* [27, 28]. The *B. subtilis* CMB32 strain synthesizes lipopeptides responsible for the antifungal activity against *Colletotrichum gloeosporioides* which causes anthracnose in plants [29]. In the strains we studied, the antifungal metabolites, unlike the known lipopeptides [30], appear to have a non-peptide nature.

Thus, *Bacillus subtilis* BZR 336g and BZR 517 strains synthesize a significant amount of fungitoxic metabolites. Thin-layer chromatography, bioautography, detection with developing reagents and optical spectroscopy methods allowed for data directly or indirectly characterizing the properties and structure of bioactive compounds produced by the studied strains. Their prospective value lies in the ability to synthesize a large set of antifungal metabolites, including, probably, non-peptide compounds. The results obtained confirm the high potential of *B. subtilis* BZR 336g and BZR 517 strains when used to develop means of protection against phytopathogenic fungi.

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