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ANTIFUNGAL ACTIVITY AGAINST PATHOGENS OF CEREALS AND CHARACTERIZATION OF ANTIBIOTICS OF *Streptomyces* sp. STRAIN K-541 ISOLATED FROM EXTREME ECOSYSTEMS IN KAZAKHSTAN

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Abstract

The general requirement for biologicals is that they must be insensitive to climate change and soil conditions, including soil physicochemical composition, fertility levels, and pH values. Actinomycetes isolated from extreme habitats are able to produce biologically active substances not only under neutral conditions but also in saline, alkaline and acidic environments, which determines their importance in the biopreparations, being developed for plant protection. This study is the first to report the *Streptomyces* sp. strain K-541 antibiosis against the causative agents of several cereal fungal infections under various environmental conditions and the identification of the antibiotic produced. *Streptomyces* sp. strain K-541 isolated from extreme ecosystems of Kazakhstan was cultured under neutral (pH 7.0) and alternative growth conditions at 25.0 g/l NaCl (pH 7.2) or 2.5 g/l Na_2CO_3 (pH 8.0). Antifungal activity was determined in agar block diffusion experiments and under paired co-incubation with phytopathogenic fungi *Fusarium solani* (Mart.) Sacc., *F. oxysporum* Schlecht., *F. heterosporum* Nees, *F. sporotrichiella* Sherb., *Piricularia oryzae* Cavara, *Alternaria triticina* Prasada & Prabhu, *A. alternate* (Fr.) Keissl., *Bipolaris sorokiniana* (Sacc.) Shoemaker, and *Aspergillus niger* van Tieghem. For antibiotic A-541 production, the strain was cultured on an orbital shaker (180-200 rpm) for 120 hours at 28 °C. The antibiotic was extracted with organic solvents and analyzed using thin layer chromatography and spectrophotometry. The studies have shown high antifungal activity of K-541 against all the phytopathogens examined. After 72 hour incubation at 25 °C the growth inhibition zones were 20-56 mm in diameter depending on growth conditions which simulated different ecological niches. In co-culturing the strain K-541 and the phytopathogenic fungi, the fungal colonies decreased 1.8-2.7 times in diameter indicating the possibility of K-541 introduction into soil biocenoses for biocontrol of cereal fungal pathogens. High inhibition of growth was also observed under saline (2 % NaCl) and alkaline (0.2 % Na_2CO_3) conditions. The antibiotic produced by strain K-541 was classified as a member of polyene group, a subgroup of the hexaenes. So strain K-541 is recognized as promising for the development of a new biopreparation with fungicidal activity against causal agents of cereal fungal infections under different environmental conditions.

Keywords: extremophilic streptomycete, antibiotic, antifungal activity, phytopathogenic fungi, wilt, rice blast disease, leaf blight, common root rot, spot blotch, mold, *Fusarium solani*, *F. oxysporum*, *F. heterosporum*, *F. sporotrichiella*, *Piricularia oryzae*, *Alternaria triticina*, *A. alternata*, *Bipolaris sorokiniana*, *Aspergillus niger*, cereal crops.

Recently, the spread of toxinogenic fungi, the causative agents of cereal crop diseases, has increased dramatically. A loss of more than 40 % grain yield results from attack of *Fusarium*, *Alternaria*, *Pyricularia* and *Aspergillus* species [1]. When infecting plants, micromycetes, along with a significant economic damage to agriculture, are environmentally harmful. However, the widespread use of chemical agents for protecting plants against fungal diseases also poses a particular danger, since the negative influence of fungicides increases with time. The

situation is worsening by the development of fungal resistance to fungicides, followed by the need to increase the dose of chemicals. In integrated plant protection, biological methods are relevant. This approach particularly includes the use of microorganisms and their secondary metabolites to create effective, environmentally friendly biofungicides with different mechanisms of action, as well as to increase plant resistance to abiotic stresses. The discovery of microorganisms forming compounds with a wide range of biological activity is of current interest, for the search for which various ecosystems are of interest, including the poorly studied extremes [2-4].

Actinomycetes are producers of secondary metabolites with antibacterial, antifungal, insecticidal, and other actions (5-7). They are an important component of microbiocenoses, and their quantitative and qualitative composition is a factor characterizing the ecological state of natural ecosystems [8]. Many types of actinomycetes, including those belonging to the genus Streptomyces, are known as antimycotic agents that inhibit pathogenic fungi [9-11]. The ability of actinomycetes to colonize the surface of plant roots, to produce antibiotics and extracellular enzymes ensures their high efficiency as biocontrol tools of plant protection against diseases [12-14].

Grain farming is the main branch of plant growing in Kazakhstan, where saline land accounts for 15.2 % of the total area of rural land. The remaining soils are also saline to varying degrees, heterogeneous in composition with low humus content [15]. Biological products for use in such difficult conditions should use microorganisms that retain biological activity in various ecological niches. Salinization of the soil and pH interfere with the protective and stimulating effect of microorganisms. Actinomycetes from extreme habitats can grow and produce biologically active substances during salinization, in alkaline and acidic environments [16, 17]. Despite the abundance of information on the biological properties of actinomycetes, their ability to regulate growth of pathogenic microorganisms, including phytopathogenic fungi, under strong salinity, high and low pH conditions has been little studied [18, 19].

In this paper, we for the first time used the biotechnological potential of extremophilic actinomycetes to develop a universal antifungal biologicals.

Our subjective was to study the antagonistic properties of the strain *Streptomyces* sp. K-541 to fungal pathogens of grain crops in different environmental conditions and to identify groups of the obtained antibiotics.

Techniques. Strain *Streptomyces* sp. K-541 isolated from an extreme ecosystem (saline solonchak, Kostanay region, Republic of Kazakhsta) was grown on three glucose-yeast nutrient media containing (g/l) glucose (2.0), yeast extract (1.0), peptone (2.0), agar (20.0), pH 7.2 (growth medium 1); glucose (2.0), yeast extract (1.0), peptone (2.0), NaCl (25.0), agar (20.0), pH 7.2 (growth medium 2); glucose (2.0), yeast extract (1.0), peptone (2.0), Na2CO3 (2.5), agar (20.0), pH 8.0 (growth medium 3). The pH value was adjusted with 0.1 N NaOH solution using a MP220 pH meter (Mettler-Toledo International, Inc., USA).

Strain K-541 was grown on three variants of agar and cultured for 7 days at 28 °C. Antifungal properties were determined by agar block technique [20]. Antifungal activity of the strain grown under neutral, saline and alkaline conditions was evaluated in tests with five genera of phytopathogenic fungi which are the causative agents of the main fungal diseases of wheat and rice. These are *Fusarium solani* (Mart.) Sacc., *F. oxysporum* Schlecht., *F. heterosporum* Nees, *F. sporotrichiella* Sherb.; *Piricularia oryzae* Cavara; *Alternaria triticina* Prasada & Prabhu, *A. alternate* (Fr.) Keissl.; *Bipolaris sorokiniana* (Sacc.) Shoemaker; *Aspergillus niger* van Tieghem. Czapek-Dox agar melted and cooled to 40-50 °C was

inoculated with a suspension of conidia of phytopathogenic fungi (108 CFU/ml, 1 ml per 100 ml medium) and poured into Petri dishes. Blocks of the grown culture were cut out with a drill (7 mm in diameter), transferred to Petri dishes pre-inoculated with test cultures of phytopathogenic fungi, and allowed for 72 hours at 25 °C. Blocks cut from pure agar media served as controls. Antagonistic activity was estimated by the lysis zone diameter of the test cultures measured with 1 mm accuracy.

For spores, strain K-541 was grown on Gause mineral salts agar 1 for 7 days at 28 °C. Spores were removed by washing. Spores (10^9 CFU/ml) were inoculated in liquid nutrient medium with oatmeal (1 ml per 100 ml). The growth medium contained (g/l) glucose (15.0), oat flour (15.0), CaCO3 (2.5), and NaCl (5.0); pH 7.0-7.2.

For antibiotics, strain K-541 was cultured in 750 ml Erlenmeyer flasks with 100 ml oat medium (a circular shaker, 180-200 rpm for 120 hours at 28 °C). Bacterial biomass was pressed to 70 % humidity, weighed and extracted with 96 % ethanol (3 ml per 1 g biomass). Antibiotic A-541 was extracted using a mechanical stirrer (RW 20 digital, IKA, Germany) for 2 hours at room temperature in a laboratory fume hood, then for 3 hours in a refrigerator. The solvent was removed under vacuum (a rotary evaporator RV 10 Basic, IKA, Germany) at 35-40 °C. The antibiotic was extracted from the culture fluid by n-butanol (pH 7.0). Extracts were fractionated on a separating funnel, evaporated under vacuum and re-extracted by ethyl alcohol.

Ethanol extracts from K-541 strain culture fluid and biomass were chromatographed on Silufol plates R, UV 254 (Cavalier, Czech Republic), Sorbfil (Sorbpolymer, Russia) and DC-Alufolien Kieselgel 60 (Merck, USA) with chloroform + ethanol (20:1, 20:7), n-butanol + acetic acid + water (2:1:1, 3:1:1), chloroform + ethyl acetate (1:1), ethanol + butanol + water (4:1:1).

Antibiotic was detected visually on chromatograms by luminescence in UV light (a UFS-254/365 irradiator, ZAO Tekhnok Scientific and Production Association, Russia) and bioavtographically with *Fusarium oxysporum* as a test organism. Zones corresponding to the position of the individual components in the chromatogram were cut out and placed on the agar with the test organism. The active zones were removed from the plates, eluted with ethanol, filtered, and the extract was evaporated. The UV and visible absorption spectra of the total preparation and its individual fractions were measured in 96 % ethanol (a Cary 60 UV-Vis spectrophotometer, Agilent Technologies, USA).

Statistical processing was performed as per V.Yu. Urbach [21]. The tables show the mean values *(M)* and standard errors of the means (±SEM).

Results. It was shown that *Streptomyces* sp. K-541 has high fungicidal effect against all studied phytopathogenic fungi (Table 1), with 30-45 mm zone when grown at neutral conditions, 40-48 mm zone at salinity, 20-33 mm at alkaline conditions against *Fusarium*; 32-35, 36-37, 20-22 mm, respectively, against *Alternaria*, 36-38, 38-40, 30 mm against *Bipolaris*, and 40, 56, and 50 mm against *Piricularia*. It should be noted that streptomycetes are widely distributed in soils of various types, and for acidic soils, species that can suppress the development of phytopathogenic fungi at low pH are also described [22, 23].

To study possible use of extremophilic K-541 strain as a biocontrol agent of fungal infections, when introdused into the soil biocenoses, the strain was cultured with phytopathogenic fungi on Czapek-Dox (Table 2, Fig.). The colonies of *Fusarium oxysporum* was 2.2-2.1 times less in diameter, of *Piricularia oryzae* — 1.8-2.7 times less, of *Alternaria alternata* — 2.2-2.4 times less, of *Bipolaris sorokiniana* — 2.0-2.2 times less, and of *Aspergillus niger* — 2.4-2.5 times less. These

data indicate a significant inhibition of fungal growth not only at normal condition but also under salinity (2.0 % NaCl, medium B) and at an alkaline pH (0.2 % Na_2CO_3, medium C).

1. Antifungal activity of *Streptomyces* sp. K-541 against causative agents of the main fungal diseases of wheat and rice, depending on the culture medium (*M*±SEM)

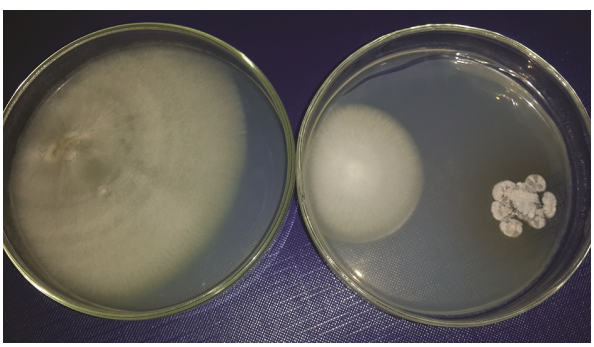
Fungal species	Lysis zone, mm		
	medium 1	medium 2	medium 3
Fusarium oxysporum	31±0.3	42±0.2	20±0.2
F. heterosporum	40±0.3	45±0.1	28±0.4
F. solani	45±0.2	48±0.4	33±0.2
F. sporotrichiella	40±0.1	45±0.1	32±0.3
Aspergillus niger	46±0.3	50±0.3	30±0.4
Piricularia oryzae	40±0.5	56±0.1	50±0.6
Alternaria alternata	32±0.3	36±0.4	20±0.1
A. triticina	35±0.3	37±0.2	22±0.3
Bipolaris sorokiniana	38±0.1	40±0.5	30±0.6
Control (no test culture)	0	0	0

N o t e. Glucose-yeast agar growth media 1-3 are described in the section *Techniques*.

2. Antifungal effect of Streptomyces sp. K-541 in co-culture with fungal phytopathoges of wheat and rice (*M*±SEM)

Fungal species	Variant	Diameter of colonies, mm		
		medium A	medium B	medium C
Fusarium oxysporum	Control	116±0.3	112±0.2	100±0.2
	Test	52±0.1	50±0.3	48±0.2
Piricularia oryzae	Control	64±0.2	66±0.3	60±0.3
	Test	36±0.1	30±0.1	22±0.1
Alternaria alternata	Control	88±0.4	88±0.5	86±0.3
	Test	40±0.2	38±0.2	36±0.2
Bipolaris sorokiniana	Control	48±0.1	44±0.4	42±0.4
	Test	22±0.3	22±0.1	20±0.1
Aspergillus niger	Control	114±0.3	114±0.2	112±0.5
	Test	48±0.5	46±0.1	44±0.3

N o t e. Medium A is Czapek-Dox agar, medium B is Czapek-Dox agar with 2 % NaCl, and medium C is Czapek-Dox agar with 0.2 % Na_2CO_3, pH 8.0.



Inhibition of *Fusarium oxysporum* growth in co-culture with *Streptomyces* sp. K-541: on the left — control (day 10 of growth; Czapek-Dox medium, 2 % NaCl, pH 7.0).

Thin-layer chromatography indicates that antibiotic A-541 is a complex mix. The A-541-1 extracted from the culture fluid and the A-541-2 from the biomass are identic in composition. The best separation results from use of n-butanol:acetic acid:water (3:1:1) system which produces 8 individual chemical compounds with UV luminescence. This system was further used for the preparative isolation of antibiotically active fractions on silica gel plates.

Bioavtography with *Fusarium oxysporum* as a test organism showed that only components IV with Rf = 52 (antibiotic A-541-1) and Rf = 0.46 (antibiotic A-541-2) have a bioactivity in the n-butanol:acetic acid:water (3: 1: 1) extracts. The bioactive IV component produces a homogeneous band under thin-layer chromatography patterns with n-butanol:acetic acid:water (2:1:1, 3:1:1). For A-541-2, the active component was isolated from the zone with Rf = 0.46. The absorption spectra of the antibiotic complex A-541 and component IV are identical and have the main UV peaks at λ 317, 330, 354 and 376 nm. In the UV

spectrum, there are peaks characteristic of polyene antibiotics which are closest to those in the hexaen subgroup (at при 330, 354, 376 nm) [24].

Thus, the strain Streptomyces sp. K-541 is a promising agent against harmful fungal infections of grain crops (wheat and rice) due to its high antifungal activity against all studied phytopathogens. Streptomyces sp. K-541 can be introduced into soil biocenoses for biocontrol of fusarium pathogens of cereal crops in neutral and alkaline soil conditions and under salinization. This is especially valuable for crop production in Kazakhstan where the soil is heterogeneous in composition and is characterized by high salinity. The isolated antibiotic A-541 is preliminarily defined as a polyene, particularly a hexaene-type antibiotic.

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