Biosurfactants produced by the novel strain *Bacillus subtilis* H1 as an efficient tool to biocontrol fungal diseases of tomato and wheat

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Abstract. Plant fungal diseases cause up to 15-20% and, in extreme cases, up to 60% yield loss globally. The use of chemical pesticides for the suppression of fungal plant diseases has many negative consequences for the environment. Therefore, new alternatives to suppress fungal pathogens are actively sought. In this present study, biosurfactants produced by the novel strain of *Bacillus subtilis* H1 were monitored for their ability to inhibit the growth of phytopathogens *Fusarium oxysporum f. sp. lycopersici* and *Alternaria* spp in *in vitro* and *in vivo* experiments. In the *in vitro* experiment, the treatment of tomato leaves with a biosurfactant fully inhibited the growth of *F. oxysporum,* and reduced the growth of *Alternaria* spp mycelium by 11.5 times at a concentration of 1000 mg/l. In the *in vivo* experiment, the use of a biosurfactant reduced the degree of damage to tomato and wheat plants, but less than in the *in vitro* experiment. In the case of tomato leaves infected with *F. oxysporum* and *Alternaria spp* and treated with 1000 mg/l biosurfactants, a decrease of 2.6 and 2.1 times was determined relative to infected but intreated leaves. For wheat leaves, the decrease was by 1.6 and 2.0 times, respectively. It can be concluded that biosurfactants produced by *B. subtilis* H1 are promising to be used for fungal pathogens biocontrol.

1 Introduction

Plant diseases caused by various organisms - fungi, bacteria, viruses, nematodes and protozoa - affect agricultural production and lead to significant crop losses. Fungal plant diseases cause up to 15–20%, and in extreme cases even by 60% loss of yields worldwide [1]. The most widespread fungal pathogens of agricultural plants are representatives of the genera Fusarium, Alternaria, Microdochium, Phytophthora, etc. They cause not only wilts, color and shape changes, rotting, wounds, and wilting but also contaminate harvesting grains, fruits and vegetables with mycotoxins [2].

Wheat (*Triticum aestivum L.*) holds the first place in world agriculture in terms of sown area and gross harvest. This crop is harvested on all five continents in huge areas, and because of such a domination of wheat in ecosystems, wheat pathogens are highly widespread and hardly eliminating. Fungi of *Fusarium* genus reduce wheat yields by 50%

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or more. Tomatoes are one of the most demanded and cultivated crops, both on the fields and in greenhouses, the yield of which depends on many factors. In world technologies for growing tomatoes, an important role is assigned to safe methods of protection, not only in relation to human health, but also in the environmental aspect. The most significant factor in the yield can be considered pathogens of these plants. Of the most common tomato diseases, *Fusarium* lesions (*Fusarium* species) can also be distinguished [3].

To reduce plant diseases in agriculture, various strategies are used - the use of pesticides, crop rotation, plant varieties less susceptible to pathogens, etc. However, the effectiveness of these methods is insufficient due to the survival and resistance of soil pathogens [4]. At the same time, the use of a large number and quality of synthetic pesticides has an adverse effect on the environment and living organisms. Chemical pesticides contribute to ecosystem disruption and reduced agricultural resilience [5]. The search for new methods of controlling plant pathogens is an urgent task in a changing climate and population growth.

Many living organisms are producers of surface-active substances (so called biosurfactants) that were demonstrated to possess various properties including inhibition of fungal and bacterial plant pathogens. Biosurfactants are amphiphilic substances with hydrophilic and hydrophobic properties. The main producers of biosurfactants are microorganisms (yeasts, bacteria, and some filamentous fungi). Microorganisms can produce biosurfactants with different molecular structures and surface activities. Recently, it was demonstrated that along with emulsifying and surface tension changing properties, many biosurfactants have the ability to suppress fungal and bacterial growth and therethrough might be used in plant protection.

Biosurfactants are considered as an alternative to chemical pesticides due to their higher biodegradability, low toxicity, mild production conditions. In this regard, the interest of researchers is directed to the search for microorganisms capable of synthesizing biosurfactants. Bacteria of the genus Bacillus belong to such bacteria. Many species of bacteria of the genus Bacillus have been characterized as bioprotectors, e.g. *B. simpex species*., *B. amyloliquefaciens*, *B. thuringiensis*, *B. megaterium*, *B. subtilis* and etc [6]. Further studies of bacteria of the genus Bacillus determined by their ability to produce biosurfactants of the lipopeptides class, including surfactin. Mixtures of biosurfactants produced by Bacillus have a complex composition, depending on the nutrient medium, the type of strain and the conditions of the biotechnological process [7, 8]. Therefore, despite of the fact, much is already known about biosurfactants and their properties, the investigations of novel strains and their biosurfactants mixtures' composition and properties remain highly required in order to find efficient and safe tools to protect plants from diseases.

In the present study, the antifungal properties of biosurfactants (lipopeptides) produced by *Bacillus subtilis* strain H1 isolated from the oil-contaminated soil were investigated. The properties were determined in two model systems "plant – fungal pathogen" consisting of tomato or wheat plant and two fungi *Fusarium oxysporum* and *Alternaria* spp, in *in vivo* (30 days-old plants) and *in vitro* (tomato leaves) experiments.

2 Materials and methods

Biosurfactant-producing strain *Bacillus subtilis* strain H1 was obtained from the collection of the Institute of Environmental Sciences of Kazan Federal University (Russia). The strain was previously isolated from the oil-contaminated soil. For increased biosurfactant production, *Bacillus subtilis* was cultivated in glycerol nitrate medium at 35 °C and 180 rpm for 6 days. The concentration of crude glycerol in the medium was 40 g/l. The medium except crude glycerol contained (g/l): $NaNO_3$ (4.0), $K_2HPO_4 \cdot 3H_2O$ (4.0), KH_2PO_4 (3.0), $MgSO_4 \cdot 7H_2O$ (0.5), KCl (0.5), NaCl (0.5), $CaCl_2 \cdot 2H_2O$ (0.2). Then a mixture of crude

biosurfactants produced by strain H1 was extracted by acid precipitation and purified as described by [7]. Then, the mixture of crude biosurfactants produced by the strain was extracted using acid precipitation and purified as described below. As a result, the acid precipitated fraction (APF) was obtained and used for further analyses [9].

Briefly, after cultivation, the culture was centrifuged at 8000 rpm for 20 min. The cell free supernatant was adjusted to pH 2 using 2N HCl, incubated overnight at 4°C, and centrifuged at 10,000 rpm and 4°C for 20 min. The precipitate was purified by dissolving in a $CHCl_3$:CH_3OH (2:1, v/v) mixture followed by rotary evaporating under vacuum. The resulting yield of the APF was 50 mg from 1 L of cell-free supernatant.

Fungal pathogen *Fusarium oxysporum f. sp. lycopersici* was obtained from the collection of the Department of Biochemistry and Biotechnology of Kazan Federal University (Russia). Fungal pathogen *Alternaria* spp. was isolated from a diseased tomato plant. Fungi for plant infection were grown in Czapek's liquid medium, up to a spore concentration of $1x10^5$ ml^{-1}. Spore concentration was measured using a Scepter Handheld Automated Cell Counter (Millipore, Burlington, MA, USA). The effect of the obtained lipopeptides on the development of phytopathogens was determined in vitro and in a vegetative experiment. Three different concentrations of aqueous solutions of lipopeptide were used in the experiment - 100, 500, 1000 mg/l.

In the experiment *in vitro* - sterile tomato leaves were treated with lipopeptides, then cuts were made and applied 10 µl of spore suspension with phytopathogens (variants FOX-100, FOX-500, FOX-1000 and AL-100, AL-500, AL-1000 for *Fusarium oxysporum* and *Alternaria* spp., respectively). Petri dishes with leaves was incubated for 7 days at 28ºC. The number of repetitions of each variant was not less than 3. The degree of leaf damage was expressed as the ratio of leaf damage area to the total leaf area, while leaf areas were measured using the ImageJ program.

Two plants, tomato and wheat (abbreviation T and W, respectively) were used for the *in vivo* experiment. Tomato seeds were germinated on vermiculite for 14 days. After the tomato plants and wheat seeds were planted in containers containing 10 kg of soil. The number of plants in each container was 6 pcs. The experimental conditions were the same: illuminated for 16 hours and without illumination for 8 hours, temperature 22°C, soil moisture 40% of the total moisture capacity. On the 14th day after the start of the vegetation experiment, the plants were treated with lipopeptides (1000 mg/l) until the plants were completely wet. After 72 h after treatment with lipopeptides, the plants were infected with phytopotagens. For this, liquid inoculums of two pathogenic fungi *Fusarium oxysporum* (variants FOX-1000t and FOX-1000w for tomato and wheat, respectively) and *Alternaria* spp. (variants AL-1000t and AL-1000w for tomato and wheat, respectively) were used at spore concentrations of $1.7x10^5$ ml^{-1} and $1.1x10^5$ ml^{-1}, respectively. Two leaves of one plant were incised with a sterile scalpel and 200 µl of phytopathogen spores were applied and observed for 13 days. In the experiment, control samples without lipopeptides treatments and infections were laid, variants Ct and Cw for tomato and wheat, respectively. There were also negative controls in which the plants were infected but not treated with surfactin (variants FOXt, ALt and FOXw, ALw for tomato and wheat, respectively). In the vegetation experiment, samples were provided in which the plants were treated with a commercial fungicide, the active ingredients of which are carboxine – 198 g/l and tiram – 198 g/l, from Avgust. To determine the effect of various concentrations of lipopeptides on the degree of infection of phytopathogens, an assessment scale of leaf infection from 0 to 5 was used: 0 = no visible symptoms of the disease; 5 = leaf dead.

To determine the effect of lipopeptides, when infected with phytopathogens on the growth and development of plants, the morphometric characteristics of plants were determined - stem length, root length, plant biomass.

The Wilcoxon signed rank test was used to determine statistically significant differences ($p < 0.05$). Statistical analysis was performed in Statistica 10.0 software (StatSoft Inc., Tulsa, OK, USA). Graphs were prepared using Microsoft Excel 2016 MSO (Microsoft, Redmond, WA, USA).

3 Results

3.1 In vitro estimation of biosurfactants ability to suppress fungal growth

When tomato leaves were artificially infected with two phytopathogens – *Alternaria* spp. and *F. oxysporum*, fungal colony growth occurred in both cases after 5-6 days of incubation. On the 7th day of incubation, the percentage of the leaves covered by the fungal colonies was estimated to be 17% and 31% for *F. oxysporum* and *Alternaria* spp., respectively (Figure 1). On the tomato leaves infected with *F. oxysporum,* but treated with biosurfactants no fungal growth was observed independent of concentration. Biosurfactant also suppressed the growth of colonies of *Alternaria* spp., but not fully – at 100 mg/l the area of the leaves covered by the fungi decreased by 18%, and at 1000 mg/l this area decreased by 28% as compared with untreated infected leaves.

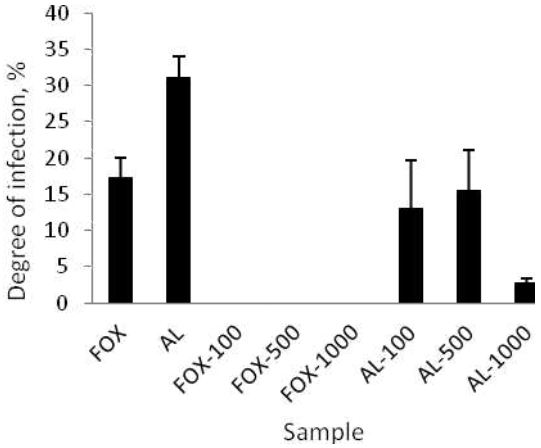

Fig. 1. The area of the tomato leaves covered by fungal pathogens (FOX - *F. oxysporum*, AL – *Alternaria* spp) colonies after artificial infection.

3.2 In vivo estimation of biosurfactants ability to suppress fungal growth

It is reported that *F. oxysporum* and *Alternaria* spp are capable of infecting not only tomato plants but a wide range of other plants such as cereals, cucumbers, cotton and etc. Therefore, on the further stage of our experiment, the ability of biosurfactants produced by *Bacillus subtilis* strain H1 was estimated *in vivo* using two types of plants: dicotyledonous (tomato) and monocotyledonous (wheat).

The degree of infection of the artificially infected plants was expressed in scores, where the maximal score 5 indicated the full necrosis of an infected plant leave. As follows from Figure 2, tomato leaves were more damaged as compared with those of wheat leaves. Thus, the scores of the leaves infected by FOX were estimated to be 4.2 and 3.5, and infected by AL – 4.7 and 4 for tomato and wheat leaves, respectively.

The highest concentration of the biosurfactant that was shown to be highly efficient in *in vitro* experiment (described above) led to suppression of the pathogens but with relatively lower efficacy – thus, FOX was suppressed by 2.6 and 1.6 times and AL was suppressed by 2.1 and 2.0 times in tomato and wheat plants as compared with corresponding negative controls (leaves that were artificially infected but not treated with biosurfactants).

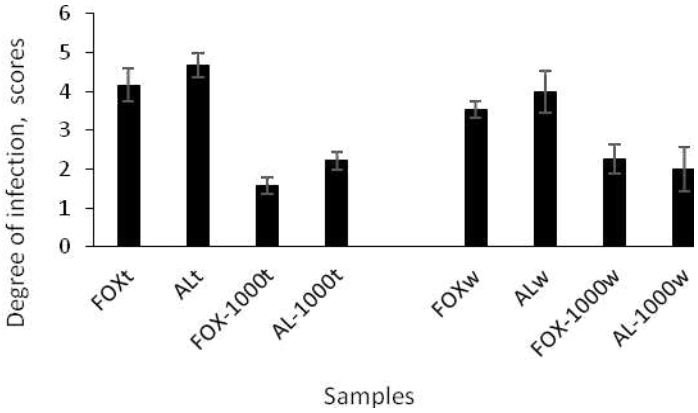

Fig. 2. Degree of infection of tomato (t) and wheat (w) plants with phytopathogens (FOX - *F. oxysporum*, AL – *Alternaria* spp) as revealed in *in vivo* experiment.

Interestingly, the artificial infection of the leaves did not lead to the infection of the whole plants. After necrosis, the leaves were defoliated and the state of the plants remained good. Indeed, the biomass of the uninfected and infected plants (with both pathogens) did not differ significantly neither for tomatoes nor for wheat plants (Figure 3). However, the effect of the biosurfactants on the biomass was revealed – it stimulated the growth of biomass by 158% for tomato and 251% for wheat.

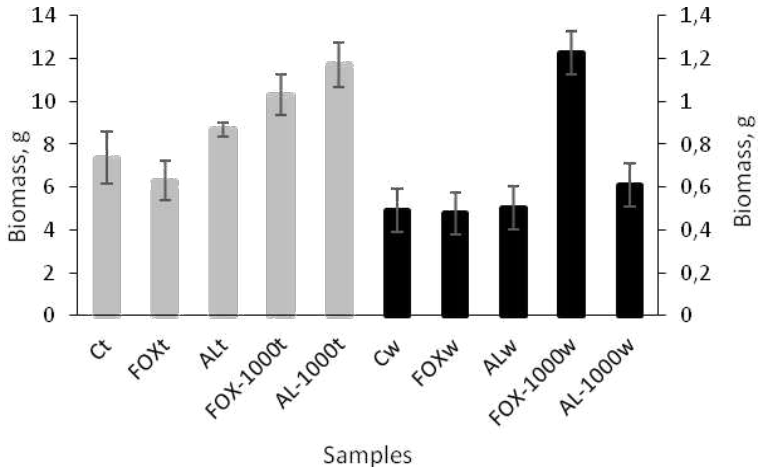

Fig. 3 Biomass of tomato (t) and wheat (w) plants infected by two fungal pathogens (FOX - *F. oxysporum*, AL – *Alternaria* spp) and treated by 1000 mg/l biosurfactants.

4 Discussion

This study explored the possibility of using different concentrations of a biosurfactant derived from *Bacillus subtilis* strain H1 as a biocontrol agent against two pathogens.

In an *in vitro* experiment, differences in the growth of mycelium on tomato leaves between *F. oxysporum* and *Alternaria* spp. were found. *F. oxysporum* and *Alternaria* spp are known to have broad substrate specificity and are capable of infecting cereals, tomatoes, potatoes, and other fruit crops. The high phytopathogenicity of *Alternaria* spp. is probably since the pathogen was obtained and identified directly from a diseased tomato plant, and *F. oxysporum* is a species from a laboratory museum. Treatment with various concentrations of biosurfactant resulted in no growth of *F. oxysporum*, regardless of the concentration of biosurfactant used.

Studies of the fungicidal properties of biosurfactants have recently been actively conducted [10]. Various types of biosurfactants are evaluated, and optimal concentrations are selected [11]. The range of studies is wide, from minimum concentrations to maximum. For example, in the work of Guillen-Navarro et all, 2023, the fungicidal nature of the biosurfactant obtained from Bacillus subtilis subsp. evaluated against ten different plant pathogens at various concentrations from 25 to 500 μg/ml and above [12]. According to the literature, biosurfactants are active at most micromolar concentrations and plant protection is induced at the highest concentrations. It is assumed that biosurfactants interact with the fraction of the lipid bilayer of plant plasma membranes and are not perceived by plant protein receptors, which promotes plant growth [13].

The maximum decrease in the growth of *Alternaria* spp. mycelium during treatment with biosurfactants was determined at a concentration of 1000 mg/l, therefore, this concentration was used in the *in vivo* experiment. It is noteworthy that compared with the in vitro experiment, in the *in vivo* experiment, the differences in the degree of infection of the leaves of control plant samples between *F. oxysporum* and *Alternaria* spp were not so significant. Infection of tomato leaves with both pathogens was higher than that of wheat leaves. Plant infection did not lead to a significant decrease in plant biomass; tomato biomass was determined at the level of 6.3 g and 8.7 g for FOXt and ALt accessions, and wheat plant biomass was 0.48 g and 0.50 g for FOXw and ALw accessions. The treatment of plants infected with phytopathogens with biosurfactants in the in vivo experiment led to an increase in biomass for all samples, except for AL-1000w. Perhaps this circumstance is associated with the ability of biosurfactants to stimulate plant growth, since in this experiment the whole plants were treated, and not just the affected areas.

Indeed, many studies have shown that biosurfactants produced by *Bacillus subtilis* are capable of inducing plant immunity under various pathosystems [14]. At the same time, it was shown that the use of biosurfactants, a group of lipopeptides produced by Bacillus subtilis, does not cause global plant defense reactions associated with large-scale genetic changes [15]. However, the mechanisms of action of lipopeptides on plant cells and the reasons for the activation of induced systemic plant resistance remain unclear.

5 Conclusion

This study shows that the use of a biosurfactant in an *in vitro* experiment resulted in the absence of growth of *F. oxysporum*, independent of the biosurfactant concentration, and reduced growth of *Alternaria spp* on tomato leaves. In the *in vivo* experiment, the treatment of plants with a biosurfactant led to a decrease in the degree of a plant infection, that however was not as significant as that in the *in vitro* experiment. Infection of leaves in the *in vivo* experiment did not lead to the defeat of the whole plant. The use of biosurfactants produced *by Bacillus subtilis* as fungicidal agents is promising.

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