Phytate-hydrolyzing rhizobacteria: abiotic stress tolerance and antimicrobial activity

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Abstract. Phytate-hydrolyzing bacteria *Pantoea* sp. 3.1, 3.2, 3.5.2, 3.6.1 and *Bacillus ginsengihumi* M2.11 were previously isolated from the soil samples of the Republic of Tatarstan. The effect of cultivation conditions on the growth dynamics as well as antimicrobial activity was determined. All four *Pantoea* strains showed optimum growth at 26 °C and 28 °C and pH 6.0-7.0. The optimum conditions for the growth of *B. ginsengihumi* M2.11 strain was determined to be 26 °C, 28 °C and 37 °C and alkaline pH 7 and 8. Salt concentration in the range of 0 to 1000 mM did not significantly affect the growth of the strains. Antagonistic activity of *Pantoea* sp. 3.5.2 was studied against phytopathogenic micromycetes, identified as *Alternaria alternata* and *Bipolaris sorokiniana*. In the presence of bacterial isolate growth of *A. alternata* was inhibited by 57% and growth of *B. sorokiniana* – by 85%. Minor growth inhibition by *Pantoea* sp. 3.5.2 of gram-negative bacteria from *Enterobacteriaceae* family was observed. The presence of fungicidal activity in the *Pantoea* strain together with its ability to hydrolyze soil phytates and overcome abiotic stress factors in soil can possibly serve as the basis for the new fungicide of microbial origin.

1 Introduction

One of the main problems of the new millennium is getting more and more agricultural products from fertile arable land, which is annually reducing per capita. The increasing public health concern, growing organic food industry, environmental pollution with pesticides, eutrophication of water reservoirs due to the massive use of mineral fertilizers, as well as significant increase in their cost have played a huge role in the development of the global biofertilizer industry. Thus, biological fertilizers and biopesticides occupy a separate unique place in the market of agricultural products all over the world today.

A significant improvement of crops growth and yield due to beneficial microorganisms is described by many authors [1-3]. However, insufficient knowledge of the basic molecular mechanisms of that interaction hinders widespread commercial use of plant-growth-promoting rhizobacteria (PGPR). PGPR are characterized by production of plant growth regulators such as indolyl-3-acetic acid (IAA), nitrogen fixation, mobilization of

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phosphorus and other macro- and microelements of the soil, and antagonism of plant pathogens by secreting siderophores, cellulases, proteases, antibiotics and cyanide, thus beneficially affecting the plant growth [2].

Increasing the availability of macronutrients for plants' nutrition is an important step in the improvement of agricultural production. In soil, the unavailable form of organic phosphorus is mainly represented by phytic acid salts – phytates. Enzymes that break down phytate – phytases – are actively secreted by some soil microorganisms and release inorganic phosphorus from insoluble phytates [4]. There is no extracellular phytase activity of plant origin in the rhizosphere, therefore, microbial phytases play crucial role in the soil organic phosphorus cycle [5].

At the same time, the acute problem in agriculture is the fight against plant diseases caused by phytopathogenic microorganisms. Phytopathogenic micromycetes are capable of releasing biologically active substances that damage plant tissues by slowing down the respiration, reducing photosynthesis, disrupting nitrogen and carbohydrate metabolism, damaging cell membranes, inhibiting mitochondrial DNA replication, and causing disruption of the mitotic cycle in meristems [6]. Moreover, bacteria are also able to cause diseases in a wide range of plants worldwide. Phytopathogenic bacteria affect food-producing plants, colonizing either their surface or tissues and causing symptoms such as blights, spots, tissue rots, hormone misbalances that globally impact plants growth and development [7].

Therefore, usage of biological preparations based on the bacteria, capable of utilizing the natural bio-resources of the soil, satisfying the nutritional needs of plants together with the protecting them from phytopathogenic microorganism is a promising approach to sustainable agriculture nowadays. Despite numerous positive effects, often the commercialization of bacterial bio-fertilizers on a large scale is limited due to the incompatibility between the results of laboratory studies and field application of the biopreparation [8]. This may be due to a number of factors, such as physicochemical properties of the soil (for example, low or high pH), interaction with other rhizospheric organisms, poor ability of the strain to colonize plant roots, environmental factors (high or low temperature, low rainfall during vegetation, high salinity of the soil and etc). The survival of bacteria in the soil, in addition to the presence of empty ecologic niche, depends on the ability of these bacteria to compete with native microorganisms well adapted to given conditions [9].

Earlier, we have isolated phytate-hydrolyzing bacteria from the soil samples of the Republic of Tatarstan, Russia and identified them as *Pantoea* sp. 3.1, 3.2, 3.5.2 and 3.6.1, and *Bacillus ginsengihumi* M2.11. The autochthony of rhizosphere microorganisms isolated from the soil of the Republic of Tatarstan prevents the disturbance of the soil agrobiocenosis. Combined with their high efficiency, that makes them a great candidate for the use as biofertilizer in agriculture in this region. In the present work, the effect of cultivation conditions at different temperatures, pH values and salinity of the medium on the growth dynamics of the isolated rhizosphere strains as well as their antimicrobial activity is determined.

2 Materials and methods

2.1 Bacterial strains and culture media

Bacterial strains used in this study were previously isolated from the soil samples of the Republic of Tatarstan according to their ability to hydrolyze phytate. *Pantoea* strains sp.

3.1, 3.2, 3.5.2 and 3.6.1 were isolated from the forest soil sample and *Bacillus ginsengihumi* M2.11- from the soil sample of the large farm complex GUP "Mayskiy". These microorganisms were maintained at 4°C on Luria-Bertani Agar (LB).

The examined fungi were cultivated in the Czapek medium [10]: Saccharose, 30.0 g; NaNO₃, 3.0 g; K₂HPO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; KCl, 0.5 g; FeSO₄·7H₂O, 0.01 g; agar, 15 g; distilled water, 900 ml; pH 4.5. The plates were incubated at 28°C for 5 - 14 days.

2.2 Effect of salt, pH and temperature on the bacterial growth

The effect of different temperature on the growth of *Pantoea* and *B.ginsengihumi* M2.11 strains was evaluated in the temperature range from 4 to 40 °C. To determine the effect of pH on the bacterial growth, pH range from 2.0 to 9.0 was used. The influence of salt on bacterial growth was determined on LB medium in the range of NaCl concentrations from 0 mM to 1000 mM.

The LB-broth was inoculated with 1% of the culture. Bacteria were grown for 48 h at 200 rpm shaking. The study was performed in three biological replicates. Growth of the cultures was evaluated by the change in optical density (OD) on a spectrophotometer (Bio-Rad, USA) at a wavelength of 600 nm every 4 hours.

2.3 Identification of micromycetes

DNA was isolated from the mycelium using the Don Liu method [11] [Liu et al., 2000]. The mycelium was thoroughly rubbed in 500 ml of TE buffer (400 mM Tris-HCl, 60 mM EDTA, 1% SDS, pH 8.0) to a homogeneous suspension and left for 15 minutes at 25°C. 150 ml of potassium acetate buffer (pH 4.8; 60 ml of 5 M of potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of distilled water) was added, suspension was shaken and then centrifuged at 11,000 rpm for 1 min. Equal volume of isopropyl alcohol was added and carefully mixed. The mixture was then centrifuged at 11,000 rpm for 2 minutes and supernatant was discarded. The resulting DNA precipitate was washed with 300 µl of 70% ethanol and centrifuged at 11,000 rpm for 1 min, the supernatant was discarded. The precipitate was dried at room temperature and dissolved in 50 µl of 1 × Tris-EDTA. Purified DNA was used for PCR.

Amplification and sequencing of ITS (internal transcribed spacer) region was performed to identify intraspecific differences in the sequences of these genes. PCR was carried out using the Taq-polymerase (Thermo Fisher Scientific, USA) with combinations of ITS1-ITS4 PCR primer pair (ITS1 dir – tccgttaggtgaacctgcgg; ITS4 rev – tcctccgcttattgatatgc) for amplification of the coding part of 5.8S rRNA ribosomal genes [12]. Samples were incubated for 4 min at 90°C, subjected to 35 cycles of denaturation (30 s at 94°C), annealing (20 s at 55°C), and extension (40 s at 72°C), followed by a final extension step (7 min at 72°C). Presence of PCR products was confirmed by DNA electrophoresis in 1% agarose gel. PCR products were purified using a PCR purification kit (Thermo Scientific, Lithuania) and sequenced at Syntol (Moscow, Russia). The analysis of 5.8S rRNA gene sequences was performed using the BLAST algorithm, a software package presented on the NCBI server (<http://www.ncbi.nlm.nih.gov/blast>).

2.4 In vitro antifungal activity assay

To determine the antagonistic activity of *Pantoea* strain against the micromycetes the agar well method was used [13]. Micromycetes were cultured on Czapek medium for 7 days. Then the suspension of mycelial and spore mass (10^5 CFU/ml) in LB broth was prepared from the micromycete colonies. *Pantoea* strain was cultured on LB-agar plates for 24 h, then 8 mm diameter well was excised from the centre of the agar plate and 200 μ l of micromycete suspension (10^5 CFU/ml) was added. Control plates without bacterial growth were prepared simultaneously. Plates were incubated at 28°C for 7 - 14 days and examined for the growth inhibition. Experiments were repeated three times. The growth inhibition of the test fungi was calculated using the following formula:

$$\text{Inhibition (\%)} = (R - r) / R * 100,$$

where R — (a control value) represents the radial growth of fungus in control sets.

r—the radial growth of the fungus in sets with bacteria.

2.5 Screening of antibacterial activities of isolates

2.5.1 Perpendicular streak method

Screening for the antibacterial effect of *Pantoea* sp. 3.5.2 was carried out by perpendicular streak method of Madigan et al. [14] against the following strains: *Serratia marcescens*, *S. grimesii*, *Morganella morganii*, *Erwinia* sp., *Pantoea agglomerans*, *E. coli* dH5a, *Salmonella enterica* and *Bacillus pumilus*. A single streak of the pure producer strain was inoculated in the middle of the assay LB-plate. The plates were incubated for 4 days at 37°C. After the incubation period, test organisms were cross-streaked along the line of grown isolate and finally the plates were incubated for 1–2 days at 37°C. Each streaking was started near the edge of the plates and streaked toward the *Pantoea* growth line. The microbial interactions were analysed by evaluating the zones of inhibition measured in mm.

2.5.2 Preparation of cellular extract

Cells were grown in LB medium at 37°C for 20 h and harvested at 4°C by centrifugation for 10 min at 8000 g. Cells were then washed 3 times with 20 mM sodium acetate buffer, pH 4.5, and lysed 3 times by repeated freezing at 80°C for 15 min and thawing at room temperature for 20 min. After being resuspended in 20 mM sodium acetate buffer, pH 4.5, cells were sonicated on ice 10 times for 10 s with 30-s intervals. Cellular debris was removed by centrifugation at 4°C for 30 min at 18,000 rpm.

2.5.3 Determination of Minimum Inhibitory Concentrations (MIC)

The inhibitory potential of bacteria growth by culture liquid and cell extract of *Pantoea* sp. was determined by broth microdilution method in 96-well microtiter plates [15]. *Escherichia coli* Dh5a and *Serratia marcescens* Sm6 strains were used as test cultures. The inoculum of the strains was diluted 1000 times in LB and cultured at 37 ° C until the OD600 0.1 (10^8 CFU / ml) was reached. For the experiment, microbial cultures were diluted to a concentration of 1000 CFU / 10 μ l. Wells of a 96-well plate were treated with sterile 0.1% BSA for 10 min at room temperature. 90 μ l of PBS buffer was then added to each well. As the active substance, 90 μ l of cell extract (CE) or culture liquid (CL) of *Pantoea* were added to the first wells of a row of a 96-well plate and a serial 10-fold

dilution was performed. Ampicillin (100 µg/ml) was used as a control. The plate was incubated at 37 °C for 12 hours and the results were evaluated. MIC values were defined as the sample concentration that prevented the bacterial growth [16].

3 Results and discussion

3.1. Responses of the strains to Abiotic Stress factors

One of the strategies to overcome the limitations of biofertilizers application is the use of native microorganisms adapted to the ecological conditions of each region [17]. We have isolated phytate-hydrolyzing bacteria from various agro-ecological niches and natural habitats of the Republic of Tatarstan, Russia [18, 19]. Strains were isolated on PSM solid medium, which contains calcium phytate as a sole source of phosphorus. The maximum number of phytate-hydrolyzing colonies was obtained from forest soils, which had more than 100×10^3 CFU per gram of soil. Soil of large farm "Mayskiy" contained 29×10^3 colony forming units (CFU) per gram of soil. Isolates were identified as *Pantoea* sp. 3.1, 3.2, 3.5.2, 3.6.1 and *B.ginsengihumi* M2.11.

Plant growth is strongly influenced by many biotic and abiotic factors, which limit the use of biofertilizers [7]. Investigation of the role of temperature, pH and salinity on the growth of isolated soil bacteria was performed. All four *Pantoea* strains showed similar response to tested factors, so the average data is present on the figures.

We examined the effect of temperature in the range of 4-40 °C on the growth dynamics of *Pantoea* strains (Fig. 1A) and *B. ginsengihumi* M2.11 strain (Fig. 1B). The optimum temperatures for the growth of *Pantoea* strains were 26 °C and 28 °C (Fig. 1A) – cultures reached the maximum level of biomass compared to growth at other temperatures. Bacteria entered the prolonged stationary phase at 12 hour of growth. Growth curve of *Pantoea* strains at 37°C was comparable or even better at the initial stage of growth, but after 16 hours a sharp decrease in the level of biomass accumulation occurred. This is explained by the fact that the temperature typical for the body of mammals (around 37 °C) is not optimal for the growth of strains isolated from soil ecosystem. At 4 °C the ability to grow was practically inhibited. *B. ginsengihumi* M2.11 strain showed same high level of biomass accumulation at 26 °C, 28 °C and 37 °C (Fig.1B). Bacteria entered the stationary phase only at 20 hour of growth. Growth at 4 °C was almost inhibited and significantly reduced at 40°C. The obtained data indicate that all tested *Pantoea* strains and *B. ginsengihumi* M2.11 strain are mesophilic.

Fig.1. Growth dynamics of *Pantoea* sp. (A) and *B. ginsengihumi* M2.11 (B) strains at different temperatures.

The effect of medium acidity on the growth of isolated bacterial strains was studied (Fig. 2). It was shown that all *Pantoea* strains were able to grow in the range of pH values from 6 to 9 (Fig. 2A). The response to cultivation under acidic pH conditions (2.0–5.0) was expressed by almost complete growth inhibition. The optimal pH for the cultivation of *Pantoea* strains was pH 6.0–7.0. The biomass accumulation of the *Pantoea* strains at pH 9.0 was lower than at optimal pH values. *B. ginsengihumi* M2.11 demonstrated great growth ability over a broad pH range, ranging from pH 6.0 to pH 9.0. There was inhibition of growth at acidic pH values (pH 2.0–5.0). The bacteria showed optimum growth at pH 7.0–8.0 (Fig. 2B).

Fig.2. Growth dynamics of *Pantoea* sp. (A) and *B. ginsengihumi* M2.11 (B) strains at different pH values of the medium.

Soil salinity plays an important role in the microbial selection process [20]. Growth of all *Pantoea* sp. and *B. ginsengihumi* M2.11 strains in the nutrient broth with variations in salt concentration from 0 to 1000 mM showed a high tolerance capacity of the strains to high salt levels in the medium (Fig. 3). At the early stage of growth (4h) NaCl concentrations higher than 500 mM held back the growth of bacteria. Nevertheless, by the 24th hour of cultivation the level of biomass accumulation by all tested isolates remained high at all salt concentrations (Fig. 3). Thus, salt concentration of the medium did not significantly affect the growth of neither *Pantoea* strains nor *B. ginsengihumi* M2.11. Still, *Pantoea* strains showed slightly higher salt tolerance than *B. ginsengihumi* M2.11.

Fig.3. Effect of different concentrations of NaCl (mM) on the growth of *Pantoea* sp. (A) and *B. ginsengihumi* M2.11 (B) strains at 4 h and 24 h of incubation.

3.2 Identification of micromycetes and antagonistic activity of *Pantoea* sp. 3.5.2 in dual culture

Antagonistic activity of *Pantoea* sp. 3.5.2 was studied against phytopathogenic micromycetes provided by the Tatar Research Institute of Agriculture of the Agricultural Academy. Two fungal isolates were isolated from the infected plants. For precise genetic identification of fungal pathogens we used molecular genetic markers representing nucleotide sequences of the ITS regions [12]. A significant part of the ITS regions is represented by conserved sequences with identical nucleotide composition in various microorganisms, and species-specific variable regions, which allow to identify the species by comparing these sequences with those annotated in the databases [21]. Amplification of the 5.8S rRNA gene of isolated micromycetes was performed with standard primers ITS1 and ITS4 (Fig. 4).

Fig. 4. PCR amplification of ITS (internal transcribed spacer) region of the coding part of 5.8S rRNA ribosomal genes. Lane M 34: molecular weight markers, 1000 bp, Lane1 – Isolate 1, Lane2 – Isolate 2.

Sequencing of amplified ITS regions allowed to identify the high homology of isolates to the *Alternaria alternata* KS44T14 and *Bipolaris sorokiniana* HP-1744 - the identity of the sequences was 99%. Due to their wide host range and worldwide distribution, *Alternaria* species cause severe economic problems. *A. alternata* is able to produce diverse phytotoxins. *A. alternata* infection in leaves induces rapid lipid peroxidation, accumulation of hydrogen peroxide (H₂O₂), and cell death [22]. *B. sorokiniana* is the causal agent of common root rot, leaf spot and seedling blight, head blight of wheat and barley and black point of grains [23]. Both fungal isolates possess high risk for the quality and yield of economically valuable cultures. Therefore, constant search of new antagonistic bacteria against these fungi is performed.

Pantoea strain 3.5.2 was screened for antagonistic activity by measuring the inhibition zones present after 7 days of dual culture with phytopathogenic fungi. Using the Petatan-Sagahon well method on LB agar medium we showed that *Pantoea* sp. 3.5.2 had the ability to inhibit the growth of both *A. alternata* and *B. sorokiniana* (Fig. 5). Growth of *A. alternata* was inhibited by 57% in the presence of bacterial isolate and growth of *B. sorokiniana* – by 85%. In contrast, no inhibition zone was observed in the control plate, where fungal mycelia covered almost the entire plate surface (Fig. 5).

Fig.5. Antagonism of *Pantoea* sp. 3.5.2 against fungal phytopathogens - *A. alternata* and *B. sorokiniana*. Growth inhibition of fungal mycelia was examined in dual culture assay on agar plates.

Thus, we observed changes in the mycelium morphology caused by bacteria in comparison to the control – irregular and distorted shape appeared. Such alteration of mycelium morphology in the presence of bacterial metabolites was reported by many authors [24], [25]. Antifungal action of *Pantoea* may be a result of disruption of fungal cell wall or inhibition of normal conidia development [26]. It was shown, that *Pantoea* strain produced compounds with fungicidal activity against *Alternaria* and *Bipolaris* genera during its growth. The presence of fungicidal activity in the *Pantoea* strain together with its ability to hydrolyze soil phytates and overcome abiotic stress factors in soil can possibly serve as the basis for the new fungicide of microbial origin.

3.3 Antibacterial Activities of *Pantoea* sp. 3.5.2

Isolated *Pantoea* sp. 3.5.2 strain was screened for its antibacterial activity on LB-agar medium using streak-plating technique (Fig. 6). No inhibition zones were detected between *Pantoea* sp. 3.5.2 and three test-strains: gram-positive *Bacillus pumilus* 3-19 strain and the strains of the same genus as the producer-strain – *P. ananatis* Cl-18 and *P. agglomerans* U2-22. Minor growth inhibition of gram-negative bacteria from *Enterobacteriaceae* family – *S. enterica*, *S. marcescens* Sm6, *S. grimesia*, *M. morganii*, *E. coli* Dh5a – was observed (Fig. 6).

Fig. 6. Streak-plating technique to screen the antibacterial activity of isolated *Pantoea* sp. 3.5.2.: 1 – *S. enterica*, 2 – *S. marcescens* Sm6, 3 – *S. grimesia*, 4 – *Bacillus pumilus* 3-19, 5 – *M. morganii*, 6 – *E. coli* Dh5a, 7 – *P. ananatis* Cl-18, 8 – *P. agglomerans* U2-22.

To determine the minimum inhibitory concentration of the antimicrobial compound synthesized by *Pantoea* sp. 3.5.2, two bacterial cultures were used: *E. coli* Dh5a and *S. marcescens* Sm6. Since the localization and nature of the antimicrobial compound is unknown, we studied both culture liquid (CL) and cell extract (CE) of the strain for antibacterial activity. The growth of *E. coli* was suppressed by CL at the concentration of 10^{-5} , and the growth of *S. marcescens* – in the concentration of 10^{-7} . While studying the effect of the CE of *Pantoea* sp. 3.5.2 on bacterial growth, it was found that MIC to inhibit the growth of *E. coli* and *S. marcescens* is 10^{-9} and 10^{-8} , respectively. The inhibitory concentration of the control antibiotic (ampicillin), causing complete inhibition of bacterial growth, was 10^{-5} (16 µg/ml) for *E. coli* and 10^{-7} (12 µg/ml) for *S. marcescens*. In the control rows, microorganism growth was observed in all wells (Fig. 7).

Fig.7. Checkerboard microtiter plate assay testing the antibacterial activity of culture liquid (CL) and cell extract (CE) of *Pantoea* sp. 3.5.2 against *E. coli* and *S. marcescens*.

Thus, it was found that the antibacterial compound of *Pantoea* sp. 3.5.2 is present both in the culture fluid and in the cell extract of the strain.

4 Conclusion

Phytate-hydrolyzing bacteria *Pantoea* sp. 3.1, 3.2, 3.5.2, 3.6.1 and *Bacillus ginsengihumi* M2.11 were previously isolated from the soil samples of the Republic of Tatarstan. In order to understand their competitive ability in soil and survival under different environmental conditions we studied the effect of abiotic factors on their growth. As a result of experiments, we were able to demonstrate that all four *Pantoea* strains showed optimum growth at 26 °C and 28 °C and pH 6.0-7.0. The optimum conditions for the growth of *B. ginsengihumi* M2.11 strain was determined to be 26 °C, 28 °C and 37 °C and alkaline pH 7 and 8. Salt concentration in the cultivation medium in the range of 0 to 1000 mM did not significantly affect the growth of neither *Pantoea* strains, nor *B. ginsengihumi*. Antagonistic activity of *Pantoea* sp. 3.5.2 was studied against phytopathogenic micromycetes, identified as *Alternaria alternata* and *Bipolaris sorokiniana*. Growth of *A. alternata* was inhibited by 57% in the presence of bacterial isolate and growth of *B. sorokiniana* – by 85%. Minor growth inhibition by *Pantoea* sp. 3.5.2 of gram-negative bacteria from *Enterobacteriaceae* family – *S. enterica*, *S. marcescens* Sm6, *S. grimesia*, *M. organii*, *E. coli* Dh5a – was observed.

Thus, biological preparation based on phytate-hydrolyzing bacteria may be used as a new environmentally friendly biofertilizer, capable of utilizing the natural bio-resources of the soil, satisfying the nutritional needs of plants and protecting them from phytopathogenic micromycetes.

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