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Research Article

The stimulatory effect of Pantoea sp. 3.5.1 on the seed germination, plant growth and development

L.R. Nigmatulina, A.D. Suleimanova, I.B. Chastuchina,

L.R. Valeeva, M.A. Startseva, and M.R. Sharipova.

Institute of Fundamental Medicine and Biology,
Kazan Federal University,
Kazan, Republic of Tatarstan, Russia

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ABSTRACT

Recent years increasing pressure on the environment is extremely exacerbated by the increasing growth of the world population needs. According to world population prospects data [World Population Prospects: The 2015 Revision] human population will increase and by 2025 will estimate to reach approximately 8 billion people. That is why people needed to use herbicides, pesticides and fertilizers to increase agricultural productivity. But most of them are persistent environmental pollutants because of their resistance to degradation and remaining in the environment for years. It may accumulate to biohazard concentrations what leads to environment poisoning [Ritter et al., 2007]. At the same time application plant-growth promoting bacteria is environmentally friendly approach. Here we show that Pantoea sp. 3.5.1 capable to promote a strong plant growth. Also here we characterize main Pantoea sp. 3.5.1 plant growth promoting mechanisms. Thus Pantoea sp. 3.5.1 is discussed to show a great promise in development of environmentally save fertilizers.

Keywords: The stimulatory effect of Pantoea sp. 3.5.1 on the seed germination, plant growth and development, plant growth promotion, Pantoea sp. 3.5.1, phytase, indole acetic acid.

1.INTRODUCTION

One of the current trends in the development of organic agriculture is the creation of microbial biotechnologies contributing to the intensification of agricultural production and the conservation of soil fertility. It is known that soil microorganisms actively interact with plants and can have both positive and negative effects on their growth and nutrition [Egorshina *et al.*, 2011]. There are many mechanisms of positive effects of associative rhizosphere bacteria on plants. Such mechanisms include the fixation of atmospheric nitrogen, production of biologically active substances, stimulation of the soil nutrient consumption by the roots, plant pathogens biocontrol, and induction of systemic plant resistance. Based on the knowledge about the interaction of plant-bacterial associations and the examples of plant growth and nutrition improvement by inoculation of useful forms of bacteria and micromycetes, the biotechnologists have been constantly searching for microorganisms in order to create new agrobiologic drugs on their basis. The plants emit various organic compounds to the environment, such as sugar, organic acids, nucleotides, amino acids, vitamins, growth promoters, being an easily accessible and diverse food substrate for microorganisms [Mehta *et al.*, 2001.]. Along with harmless microorganisms, there are phytopathogenic bacteria that cause poisoning and diseases of plants [Zelicourt *et al.*, 2013]. The soil is a favorable environment for the development of rhizosphere microorganisms [Barassi et al.,

2007]. Calculations show that 1g of soil contains $1x10^9$ bacteria, $1x10^5$ fungi, $1x10^5$ actinomycetes, and $1x10^3$ algae. The total mass of microbial cells in the plow layer is about 6-7 tons per 1 ha. The microflora of the rhizosphere, taking part in the processes of transformation of organic matter in the soil, provides plants with essential mineral elements and biologically active substances [Pinton *et al.*, 2007]. Synthesized by microorganisms vitamins and growth substances (gibberellin, heteroauxin) have a stimulating effect on the growth processes of plants [Jana *et al.*, 2010]. Auxins, as it is known, initiate the elongation of roots and development of lateral roots and root hairs, which can be important for accelerated growth, nutrient intake and plant resistance to stress [Fowler and Thomashow 2002, Spaepen *et al.*, 2007]. Rhizobacteria, synthesizing IAA from tryptophan via indolyl-3-pyroracemic acid and indolyl-3-acetaldehyde, belong to genera *Azotobacter, Azospirillum, Enterobacter, Klebsiella* etc. The epiphytic and rhizosphere microflora of plants plays a pivotal role in the conversion of tryptophan contained in root exudates, and in PAA [Narula *et al.*, 2009]. The most effective producers of auxins are found among microorganisms living in the plant rhizosphere and phyllosphere. The ability of rhizosphere bacteria to dissolve the hard soil phosphorus-containing compounds is also considered as an important mechanism of positive effect on phosphorus nutrition of plants [Weller *et al.*, 2002]. Natural mechanisms of microbial transformation of the phytates are quite diverse. For instance, the inorganic phosphates are dissolved under the action of their secreted organic and inorganic acids, hydroxyl and carboxyl groups, which form complexes with cations of calcium, aluminum and iron, which changes the acidity of the soil [Mukhametzyanova, 2012]. In this context, the objective of the research was to study the mechanisms of plant growth-promotion by the strain *Pantoea sp.* 3.5.1

2.MATERIALS AND METHODS:

2.1. Baterial strain and plant

Pantoea sp. 3.5.1 strain were used for experiments, strain was isolated from Tatastan Republic soils in Russia and was tested by different methods. Soft spring wheat (Triticum eastivum,cv. Simbircit) was used for the bacterial inoculation experiments. Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 2010.

2.2. Nutritional medium and culture conditions

*Pantoea sp. 3.5.*1 strain were cultured at the Luria Bertani medium with 1% agar addition [Sambrook e*t al.*,1989]. The ability of bacteria to dilute hardly accessible inorganic phosphates were obtained at NBRIP medium [Nautiyal et al., 1999]. To solid NBRIP medium added 3% agar. The ability of bacteria to hydrolyze phytate were investigated on PSM medium [Sasirekha et al., 2012]. To solid PSM medium added 3% agar.

Bacteria were cultivated in glass bottles with 200 rpm shaking at the 37C° (“B. Braun”, Germany). In experiment were took 12-hour inoculate. Medium without inoculate were used as control.

2.3 Phosphate mobilizing activity assay

The ability of bacteria to dissolve inorganic phosphates characterized during the cultivation in liquid NBRIP medium [Nautiyal et al., 1999]. 12-hours inoculate were added to NBRIP medium and cultivated at 37ºC during 4 days. Medium without inoculate were used as control. Results were registered every 24 hours. Medium pH value and changing of free phosphate level were estimated during the bacteria growth.

2.3.1 Free phosphate content determination

Determination of free phosphate content investigated according to Greiner method (2004). It is based on the ability of inorganic phosphate to form phosphonomolibden ammonium in acid conditions. To determine the concentration of free phosphate calibration graph were made.

2.4. Preparation of cell lysate

To obtain bacterial cell lysate using freeze-thawing technique with sonication. Precipitated cells were freeze-thawed three times using

liquid nitrogen and thawed at room temperature. After that cells were suspended in 20 mM Na-acetate buffer, pH 4,5 and sonicated (20-50 kHz) for 10s, with an interval of 30s ten times. Then lysocyme was added at a concentration of 1 mg/ml and incubated for 30 min at room temperature. The cell lysate was centrifuged for 30 min at 15 000g.

2.5 Sterilization of seeds

Seeds were sterilized according to standard procedures [Kai et al., 2005].

2.6 The influence of the intracellular and extracellular metabolites Pantoea sp. 3.5.1 on seed germination

We studied the influence of the culture liquid and the cell lysate on wheat seeds Simbirtsit (Tatarstan republic standard received from the Niva Institute). Bacterial cultures were grown on medium LB during 22-23 hours then culture liquid were collected sterilely by centrifugation for 10 min 8 000g. Cell lysate were prepared from the precipitated cells. Then the seeds were steeped for 2 hours in culture liquid or cellular lysate. As control seeds were incubated for 2 hours in LB or in distilled H2O at room temperature. We use three different concentrations as an experiment (Table 1).

Table 1 - Test solutions

Solution	Dilution	Volume for steeping
culture liquid	None	50 ml
	1:10	
	1:100	
cell lysate	None	10-12 ml
	1:10	
	1:100	
Control LB	None	50 ml
Control water (dH_2O)		50 ml

After 2 hour incubation, sterile seeds were transferred to glass jars with sterile cotton pad steeped with 5 ml of sterile distilled water. Seed vigor was evaluated at intervals of 24 hours during 4 days by counting the germinated seeds. Results of root length growth and dry and fresh leaf growth were assessed after 4 days. All experiments were performed in three biological replicates, the results were treated statistically.

2.5 Characterization of IAA production

To determine the IAA in the cultural liquid produced by *Pantoea sp. 3.5.1* we used method described by Tanner and Anderson. Quantitative assay of amounts of IAA was performed using Gordon and Weber method [Gordon & Weber, 1951]. IAA secretion was determined by periodic cultivation mode in the liquid 10 times diluted LB media (referred to as - dLB) which contain 0.04% tryptophan. To determine the effect of the media pH on IAA synthesis bacteria were cultured on LB media with different pH value (5.0 and 7.0).

The level of production of IAA were controlled during 3 days. Every 24 hours aliquots were taked cells were pelleted by centrifugation at 14,000g for 5 minutes.

To 1 volume of the supernatant was added 4 volumes Salkovsky reagent (0.05 M FeCl3 in 35% perchloric acid). Absorbance was measured after 30 minutes at 530 nm. Determination of IAA level in supernatante carried out using a calibration curve using synthetic IAA solution. For the statistical analysis of experimental data used Microsoft Excel program, Student criterion.

3. RESULTS AND DISCUSSION

1. Phytate mobilizing activity of *Pantoea sp. 3.5.1*

The insoluble phosphates are widely distributed in the environment and are poorly accessible for plants, phosphorus-accumulating compounds. Phosphorous share in organic compounds is 30%-50% of the total soil phosphorus [Dai et al., 2011]. Phytic acid and its salts - phytates, are the main and most common form of soil organic phosphorus [Kartsev 2003]. The phosphoric acid

residues making part of phytate are chemically active and capable of binding metal ions - calcium, sodium, potassium, zinc, copper. Phytic acid can also interact with amino acid residues, transforming them into the unavailable for plant condition [Onyango and Adeola 2011]. A strain *Pantoea sp.* 3.5.1, able to grow and form the hydrolysis zones in the plates containing calcium phytate as the sole source of phosphorus, was isolated and identified from the samples of forest soil of the Republic of Tatarstan (Figure 1).

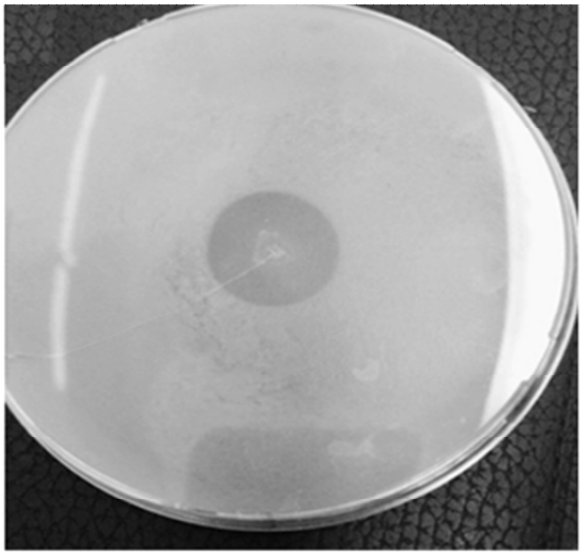
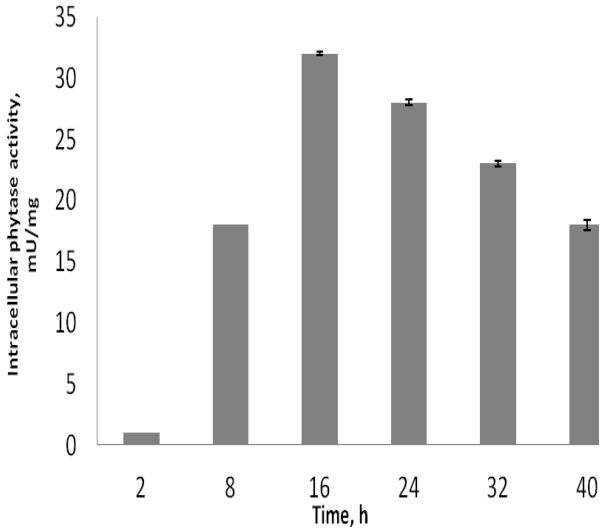


Fig. 1. Calcium phytate hydrolysis by *Pantoea sp. 3.5.1*.

To study the localization of phytase, we examined the level of phytase activity in cell fractions (culture fluid, periplasma, membrane, cytoplasm) of strain *Pantoea sp.* 3.5.1.

The maximum activity was detected in the periplasm fraction. The other cellular fractions of strain, as well as the culture fluid showed phytase activity found in traces (Figure 2). The findings showed that the phytase *Pantoea sp.* 3.5.1 is a periplasmic enzyme and its final destination of functioning is periplasm cells.

The study of the dynamics of growth and accumulation of phytase activity *Pantoea sp.* 3.5.1 on LB medium has shown that the activity of the enzyme occurred in the cell lysate during the 2nd hour of culture growth and maintained throughout all phases of bacterial growth. However, the level of accumulation was not changed significantly after cells reached the stationary growth phase of the culture during 16th hour of culture (Figure 2).



2. *Pantoea sp. 3.5.1* can change the pH value during the cultivation

It is known that organic acids produced by soil microorganisms promote transition of mineral and organic phosphates into the soil solution, after which the enzymatic release of the phosphate group by phosphatases becomes possible [Lambers, 2010].

The culture liquid of the strain *Pantoea sp.* 3.5.1 had no phytase activity detected, however, the bacteria were able to grow on a medium with phytate as a sole phosphorus source, and thus had the ability to hydrolyse the phytate. We studied the changes in the pH value during the culture of the strain on liquid PSM medium. The bacterial growth-free medium was used as negative control.

24 hours of culture at 37°C, the pH of the *Pantoea sp.* 3.5.1-containing medium decreased from pH 7.0 to pH 3.58, while the pH in the negative control showed insignificant changes - from 7.0 to 6.37 (Figure 3A). No further decrease in the medium pH was observed during culture.

It was established that there was a gradual medium acidification in the process of culture; the medium pH reached its minimum value (pH 3.58) during 12th hour of culture growth, and remained further at constant level (Figure 3B).

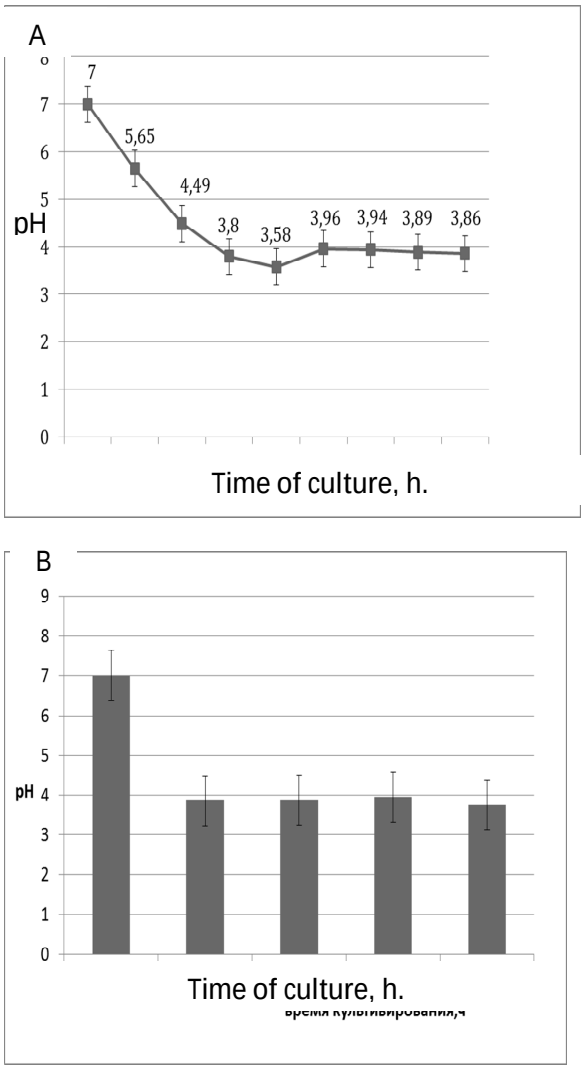


Figure 3. pH value variation during culture during first 24 h of culture (A), during 94 hours of culture (B). The ability of microorganisms to the acidification of the culture medium is associated with the synthesis of organic acids. Transformation of non-soluble phytate complexes into soluble state is the result of pH decrease during the synthesis of organic acids by bacteria [Fankem, 2006].

Thus, the formation of hydrolysis zones on a solid medium by strain *Pantoea sp.* 3.5.1 is due to the acidification of the medium around the colony and transformation of insoluble phytate complex to soluble state suitable for the phytase action.

An increase in free phosphates in the soil should have a positive influence on the growth and development of plants [Khalil 2013], due to the fact that the deficiency of phosphorus is the limiting factor for plant growth [Effendy 2008]. Thus, based on the high ability of bacteria of strain *Pantoea sp.* 3.5.1 to hydrolysis, we have investigated the effect of these microorganisms on seed germination.

3. *sp 3.5.1* metabolites effect on wheat seeds and seedlings growth

At first we investigate how cultural liquid and cell lysates in 3 concentrations (without dilution, 10 times dilution, 100 times dilution) can affect seed germination rate and seedlings growth parameters

Table 2. *Pantoea sp. 3.5.1* cultural liquid and cell lysate effect on seed germination rate and seedlings growth parameters.

	Cultural liquid concentrated (% from control)	Cultural liquid 10 times dilution (% from control)	Cultural liquid 100 times dilution (% from control)	Cell lysate concentrated (% from control)	cell lysate 10 times dilution (% from control)	cell lysate 100 times dilution (% from control)
Germinated seeds	96.51163±0.25	96.0396±1.14	93.26923±0.96	95.79±0.85	95.41284±2.08	96.15385±0.76
Average root length	114.7671±3.46	93.675±1.34	111.1624±1.89	146.685±2.15	132.6615±1.38	116.0045±2.54
First leaf length	149.0241±0.87	102.552±4.87	122.25±1.44	173.326±2.52	167.893±0.84	149.89±1.58

We have shown that treatment of wheat seeds with *Pantoea sp.* 3.5.1 culture liquid improves germination by 4-6% as compared to controls (Table 2).

In addition, we have studied the effect of the bacterial cells on the seed germination. The distilled water and the LB culture medium were used as control in order to ascertain the effect of culture medium (without bacteria) on wheat seed germination and seedling growth. The study of the effect of living bacteria on the seed germination energy revealed no germination-promoting effect.

Table 3. *Pantoea sp. 3.5.1* living cells effect on seed germination and seedlings growth parameters

	Control (distilled water) (% from control)	**Control (LB medium) (% from control)**	***Pantoea sp. 3.5.1* living cells (% from control)**
Average root length	100	101.403±3.01	114.206±0.88
First leaf length	100	117.74±1.27	127.447±2.77

We have studied the effect of culture liquid and cell lysate of *Pantoea sp.* 3.5.1 on the average length of the first leaf and the plant root. It was found that the treatment of wheat seeds with *Pantoea sp.* 3.5.1 culture liquid resulted in the increase in root length by 10-12% as compared to controls (Table 2), and the in length of the first leaf by 20-50% (Table 2).

Treatment of plants with cell lysate diluted in 1:100 ratio promoted an increase in root length by 12%; the length of the leaf increased by 49.8%. Treatment with cell lysate diluted in 1:10 ratio promoted the root growth by 25%, and the first leaf growth - by 68%. The most effective was the treatment of wheat with the concentrated cell lysate of *Pantoea sp.* 3.5.1. We noted the root growth by 36.8%, and the first leaf - by 73.3%, as compared to control (Table 2).

Thus, the cell lysate and culture liquid of strain *Pantoea sp.* 3.5.1 have a positive influence on the increase in the length of the first leaf and the plant roots, indicating the presence of biologically active metabolites, favorably influencing the growth of plants, in both bacterial fractions. Moreover, the cell lysate showed the most effective action.

Treatment of plants with living bacteria of *Pantoea sp.* 3.5.1 had virtually no effect on the growth parameters of the first leaf, however, the root length increased by 14% as compared to control (Table 3).

4. *Pantoea sp. 3.5.1 indolil acetic acid production ability.*

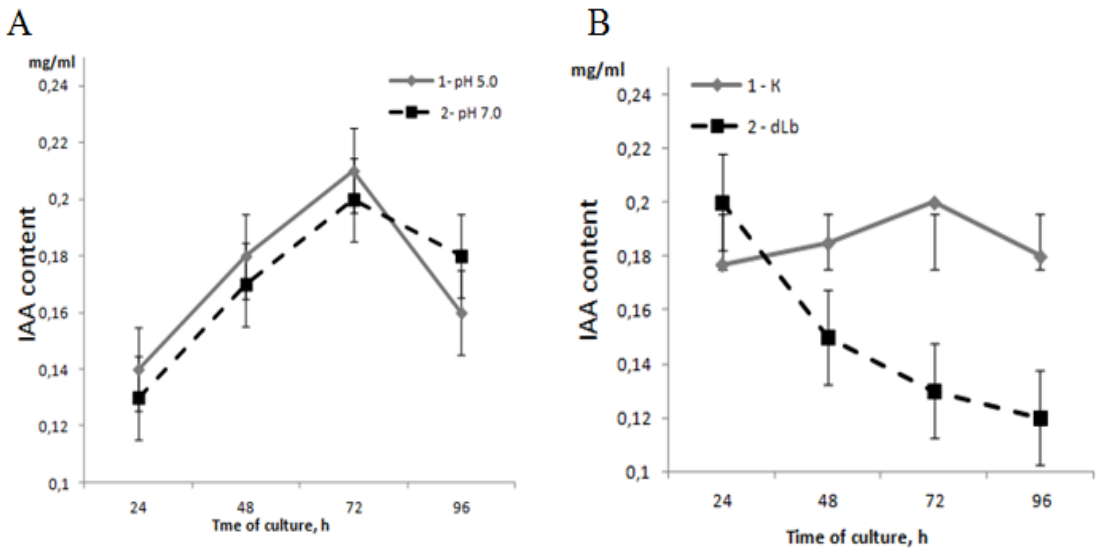


Figure 4. Pantoea sp. 3.5.1 IAA production ability in liquid culture medium A – in different pH, B-in different liquid culture medium concentrations.

The formation of plant hormones is one of the important properties of plant growth-stimulating rhizosphere bacteria. Indole acetic acid (IAA) is a phytohormone of auxin series that regulates plant growth and development. We have studied the ability of strain *Pantoea sp.* 3.5.1 to synthetize the IAA on the liquid medium. We have found that the bacteria produced IAA into the culture liquid, and maximum phytohormone formation was observed during 72nd hour of culture growth (Figure 4 A, B). Phytohormone level production at medium pH 7.0 was 10% lower than at pH 5.0 (Figure 4A). A ten-fold dilution of the culture liquid resulted in the

decreased level of phytohormone synthesis on each subsequent day (Figure 4B). This reduction of IAA synthesis could be due to the reduced growth rate of bacteria caused by 10-fold dilution of the LB culture medium. IAA production is determined by the content of tryptophan, which is an exogenous precursor of auxin synthesis in bacteria. We have shown that the bacteria of *Pantoea sp.* 3.5.1 can produce IAA into the environment and influence the plants by regulating their growth and development.

4. CONCLUSION

Thus, strain *Pantoea sp.* 3.5.1 showed a phytate-mobilizing activity, during its culture process the pH level decreased to 3.5 and the release of free phosphates in the medium increased. We have established the ability of strain *Pantoea sp.* 3.5.1 to synthesize the indolyl acetic acid in a liquid medium with its maximum at 0.21 mg/ml during 72nd hour of culture growth. Treatment of seeds with culture liquid and bacterial cell lysate resulted in the increased germination power by 6%, whereas the living bacteria had no stimulating effect on plants. We have established a positive effect of bacteria on the root system of plants. Treatment of seeds with the concentrated cell lysate resulted in the increased root growth by 36.8%, and the first leaf - by 73.3%.

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