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# Biotechnological potential of phosphate-solubilizing *Pseudomonas migulae* strain GEOT18

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**Abstract** The biotechnological potential of phosphate-solubilizing bacterial *Pseudomonas migulae* strain GEOT18, isolated from the endosphere of *Dactylorhiza incarnata* (L.) So ó (Orchidaceae Juss) was investigated. The phosphate solubilizing activity of this strain was assessed qualitatively and quantitatively by their ability to dissolve tricalcium phosphate. It was established that the *Pseudomonas migulae* GEOT18 demonstrated strong phosphate-solubilizing activity (the content of free phosphorus in the culture medium was 3.25 ± 0.05 mg/ml). It was revealed that *P. migulae* GEOT18 synthesizes indole-3-acetic acid into the culture medium up to 21.1 ± 1.4 mg/L. Furthermore, it was established that this strain produces siderophores, can fix molecular nitrogen and does not show phytopathogenic activity. It was proved that inoculation of the marigold *Tagetes patula* with the *P. migulae* GEOT18 strain increases the biomass of plants and mass of inflorescence. It accelerates the transfer to the generative phase and prolongs the duration of flowering and fruiting in comparison with non-inoculated plants.

**Keywords:** Indole-3-acetic acid, Inorganic phosphorus, Nitrogen fixation, Phosphate-solubilizing bacteria, Siderophores

## Introduction

The normal development of plants is ensured by the presence of all the essential macroelements and microelements in the required amount. Gradual soil exhaustion leads to a decrease in the content of mineral and organic substances, which requires additional soil fertilization. One of the main chemical elements for plants is phosphorus. It is involved in many vital processes, accelerates the development of plants, increases disease resistance and sugar content in fruits, enhances rooting and fruiting (Butterly *et al*., 2009). Especially, the necessity in this element is expressed at the initial period of the development when the root system is formed and during the flowering and fruit

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formation. The lack of phosphorus in plants is usually expressed in a decrease in the number of leaves and the size of the leaf blades or in their withering, decrease in the rate of tillering and rooting, retardation of fruiting, and a reduce of fruits number. The lack of phosphorus is an important factor that limits the yield of agricultural plants (Yin, 2011).

The introduction of phosphorous fertilizers does not contribute to phosphorus deficit elimination. It was found that only 10-25% of the introduced phosphorus is taken up by plants. The major part of phosphorus is flushed away from the soil or gets immobilized (Sharma *et al*., 2013).

Presently, there is a search for an alternative to mineral fertilizers that would covers the needs in phosphorus and does not exert an unfavorable effect on the environment. Such alternative can be endophytic microorganisms, in particular, bacteria and fungi. Many of them are characterized by microbiological solubilization of insoluble phosphorus into its soluble forms. The most widespread method of phosphate solubilization in bacteria is the excretion of organic acids (malic, lactic, citric, succinic, isovaleric, isobutyric, and acetic acids) (Rodríguez and Fraga, 1999). Gluconic acid, primarily excreted by *Pseudomonas* and *Bacillus*, is especially effective. It binds with tricalcium phosphate and other insoluble forms of phosphorus in the soil (iron and aluminum compounds) and chelates Ca, Fe, and Al cations, which leads to the formation of non-dissociating complexes, and the equivalent amount of phosphate moves to the soil solution.

The plant endosphere contains a great number of plant-growth promoting bacteria (PGPB). PGPB can stimulate the growth of plants and improve their productivity due to mobilization of inorganic soil phosphates, fixation of molecular nitrogen, and chelating of iron (Klopper *et al*., 1989; Fürnkranz *et al*., 2009; Babu *et al*., 2015; Hanif *et al*., 2015; Subramanium and Sundaram, 2020). The application of PGPB bacteria as inoculants showed positive results in the increase in the yield of grain cultures such as *Triticum aestivum*, *Zea mays*, *Oryza sativa*, and *Saccharum officinarum* (Mehnaz *et al*., 2001; Kim *et al*., 2011; Bhattacharyya and Jha, 2012). Thus, the search was aimed to investigate for new and more efficient strains of phosphate-mobilizing bacteria in a relevant task.

## Materials and methods

### *Bacterial strains and growth conditions*

The study included a bacterial *Pseudomonas* sp. strain GEOT18 isolated from the endosphere of *Dactylorhíza incarnata* (L.) Soó (Orchidaceae Juss.). The strain was cultivated on the LB-agar plates at 28°C.

### *DNA extraction, 16S rRNA amplification and phylogenetic analysis*

Molecular genetic identification of the strain was performed by the PCR amplification of 16S rRNA gene. Genomic DNA from the culture of bacterial cells was extracted using the bacterial genomic DNA isolation kit ExtractDNA (Evrogen) following the manufacturer's protocol. PCR amplification of 16S rRNA gene was carried using universal prokaryotic primers 356F (5'-ACWCCTACGGGWGGCWGC) and 1064R (5'-AYCTCACGRCACGAGCTGAC) according to the standard method (Sambrook *et al*., 2001).

PCR was performed with a commercial BioMaster HS-TaqPCR-Color kit (Evrogen) following the manufacturer's protocol. The conditions for PCR were as follows: an initial denaturation at 95℃ for 4 minutes, 30 cycles of denaturation at 95℃ for 1 minute, annealing at 55℃ for 1 minute, extension at 72℃ for 90 seconds, and final extension at 72℃ for 5 minutes after the cycles.

The PCR amplification products were detected by the method of gel-electrophoresis on a 1.5% agarose gel. The isolation and purification of a DNA fragment from the agarose gel were made with a commercial CleanupMini kit (Evrogen) following the manufacturer's protocol. The identification of a bacterial strain based on the analysis of the primary nucleotide sequence was performed in the GenBank database using the BLAST software. A phylogenetic tree, based on 16S rRNA sequences, was constructed by the neighbor-joining method. The sequence obtained in this study was deposited in the GenBank.

### *Phosphate solubilization assay*

Qualitative evaluation of the phosphate solubilization ability of the *Pseudomonas* sp. GEOT18 was performed on Pikovskaya agar medium, g/L: $NH_4Cl$ – 1.0, $MgSO_4 \cdot 7H_2O$ – 0.6, glucose – 10.0, $Ca_3(PO_4)_2$ – 6.0, solution of microelements – 20 ml/L, agar-agar – 20.0. The content of microelement solution, g/L: $FeSO_4 \cdot 7H_2O$ – 0.01; $CuSO_4 \cdot 5H_2O$ – 0.1; $MnSO_4 \cdot 2H_2O$ – 0.01. The results were evaluated by the size of clear halo zone of dissolved phosphate around the colony on the 3rd day of the cultivation.

Quantitative evaluation of phosphate solubilizing activity of the bacterial strain was performed according to the accumulation of free phosphorus in the liquid medium with tricalcium phosphate (TCP) with cultivated *Pseudomonas* sp. GEOT18 by the spectrophotometric method with ammonium molybdate (Murphy and Riley, 1962). The strain was cultivated in the liquid medium with the following composition: $NH_4Cl$ – 1.6 g/L, $MgSO_4 \cdot 7H_2O$ – 0.2 g/L, glucose – 20.0 g/L, TCP – 5.0 g/L, solution of microelements – 20 ml/L. The content of microelement solution, g/L: $FeSO_4 \cdot 7H_2O$ – 0.01; $CuSO_4 \cdot 5H_2O$ – 0.1;

$MnSO_4$ $2H_2O$ – 0.01. As a control sample, a sterile un-inoculated medium with the same composition was used. The samples were cultivated in flasks with 100 ml of the medium on the shaker at 150 rpm at 28°C for 12 days. For this purpoce a sample was centrifuged for 2 minutes at 8000 rpm and mixed with the molybdenum reagent ($H_2SO_4$ – 37.24 g/L; NaCl – 8.775 g/L; $(NH_4)_6Mo_7O_{24}$ $4H_2O$ – 2.348 g/L; Tween-80 – 20 ml) at the ratio of 4 ml of the reagent to 20 µl of supernatant. Absorbance was measured at $OD_{340}$ in Unico 2802S/VIS spectrophotometer. All samples were in triplicate. The amount of the released phosphorus was calculated from calibration curve of the $KH_2PO_4$ standard dilutions.

Simultaneously, the pH of the bacterial suspension were measured with pH-meter pH-150M. The number of viable bacterial cells (CFU) in the cultural liquid was estimated by the method of consecutive dilutions (Koch's method) on a meat-peptone agar medium.

### *IAA production assay*

The IAA (indole-3-acetic acid) and its intermediates was assayed based on the colorimetric method with Salkowski reagent (Tsavkelova *et al.*, 2007). The strain was cultivated in the LB medium on a rotor shaker ES-20 (Biosan, Latvia) at 180 rpm at 28°C for five days. Simultaneously, un-inoculated LB medium was incubated as control. After the cultivation, the sample was centrifuged for 15 minutes at 7000 rpm. Salkowski reagent was added to 5 ml of the supernatant at the ratio of 1:1. Salkowski reagent composition: 35% $HClO_4$ and 0.5 M $FeCl_3$ at the ratio of 50:1. After that, the flasks were placed in the incubator at 30°C for 20 minutes. The absorbance of the liquid was measured using a spectrophotometer Unico-2802S/VIS (USA) at 540 nm. The content of IAA was calculated from calibration curve of the synthetic IAA standard dilutions.

### *Capacity to siderophores production*

The siderophore production of *Pseudomonas* sp. GEOT18 was evaluated using Blue Agar CAS medium containing chrome azurol S (CAS) and hexadecyltrimethylammonium bromide (HDTMA) as indicators (Schwyn and, Neilads, 1987). The evaluation of siderophore production was made by the orange colored zone around the *Pseudomonas* sp. GEOT18 colony.

### *Biological molecular nitrogen fixation assay*

The capacity of the *Pseudomonas* sp. GEOT18 to fix nitrogen was evaluated using Jensen selective medium of the following composition, g/L:

$K_2HPO_4$ – 1; $MgSO_4$ – 0.5; NaCl – 0.5; $FeSO_4$ – 0.1; $Na_2MoO_4$ – 0.005; $CaCO_3$ – 2; saccharose – 20; agar-agar – 15 (Jensen, 1942). The strain was cultivated in an incubator at 28 °C for 4 days. The growth of bacteria on a selective medium indicated nitrogen fixing activity.

### *Assay for phytopathogenic activity*

The strain was tested for phytopathogenic activity by the presence of maceration of the plant tissue *in vitro* on a potato tuber (Hussain *et al*., 2007). Potato tubers were sterilized in 70% ethanol solution and cut into 1 cm cubes in aseptic conditions. The studied strain was placed on plant tissue and cultivated for five days at 28 °C. The presence or absence of maceration of the plant tissues was evaluated visually.

### *Plant inoculation assay*

Laboratory vegetative tests were performed for the evaluation of the influence of *Pseudomonas* sp. strain GEOT18 on the growth and development of *Tagetes patula* as a culture that is most sensitive to phosphorus deficiency. The sand for the experiment was preliminary flushed, sieved through a 2.5 mm gauge filter, and calcinated in a laboratory furnace at 200 °C. The sand was placed into pots with drainage to fill ¾ of the volume. TCP was introduced in some pots (0.5 g in each pot). The culture was introduced to the test samples (not less than $10^8$ CFU/ml). The cultivation was performed in a climatostate KS-200 16 hours day/8 hours night for 60 days. The required elements were introduced as a nutrient solution (NS) with the following composition, g/L: $Ca(NO_3)_2$ – 1; $KH_2PO_4$ – 0.25; $MgSO_4$ – 0.25; KCl – 0.125; $Fe_2Cl_6$ – 5 drops of a 1% solution. The source of phosphorus was insoluble TCP. The nutrient solution without phosphorus was used as a control. Soil moisture was maintained at the level of 60% from the maximum water-holding capacity. The energy of seed germination was evaluated on the 3rd day after the seeding and germination rate – on the 7th day after the seeding. The statistical analysis of the data was performed using ATTESTAT 12.0.5 software and the "Microsoft Excel" application.

## Results

### *Identification of the isolated strain*

A comparative analysis of the 16S rRNA gene sequence showed that the strain belongs to the genus *Pseudomonas* and show the maximum sequence

similarity 99% with *P. migulae.* This gene sequencing is deposited in GenBank accession number MT180656. The phylogenetic tree was made using the neighbor-joining method (Figure 1).

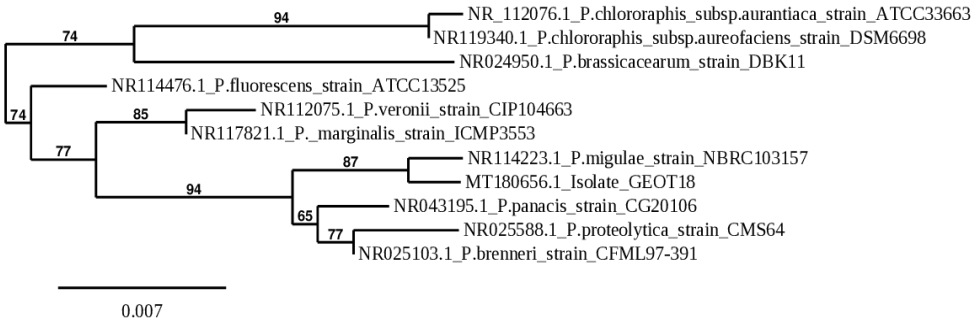


**Figure 1.** Phylogenetic tree of the isolated strain and reference species based on the 16S rRNA genes according to the neighbor-joining method

### *Phosphate-solubilizing activity of the isolated strain*

Qualitative evaluation of the phosphate-solubilizing activity in the Pikovskaya agar medium showed that the *P. migulae* GEOT18 demonstrates strong capability of solubilization with a clear halo zone diameter 25 mm (index of solubilization 5).

Quantitative analysis of phosphate-solubilizing capacity showed that the maximum free phosphorus content in the culture medium with the *P. migulae* GEOT18 was 3.25±0.05 mg/ml (Figure 2A). An increase in the concentration of soluble phosphorus was observed for 10 days, pH in the culture liquid decreased from the initial 6.9 to 3.7 (Figure 2B). The growth dynamics of the culture is presented in the graph (Figure 2C) showing that the strain characterized by an active growth for 10 days. The exponential phase of the growth was observed from the 1$^{st}$ to the 5$^{th}$ day of cultivation. The maximum number of cells was 7.2 lgCFU/ml.

### *Influence of the isolated strain on Tagetes patula growth*

The previous studies showed that inoculation of oat and barley with the *P. migulae* GEOT18 exerted a positive effect on wet and dry plant mass, development of leaves, and increase in the content of chlorophylls a and b in comparison with the control (Rassokhina *et al*., 2020). In the present study the influence of the *P. migulae* GEOT18 on the growth and development of *T. patula* was evaluated in the conditions of sand culture. Several trials were conducted to study the influence of phosphorus on the shoot length, root length,

number of flowers, wet and dry mass of shoots, roots and flowers, and the rate of flower formation (Table 1).

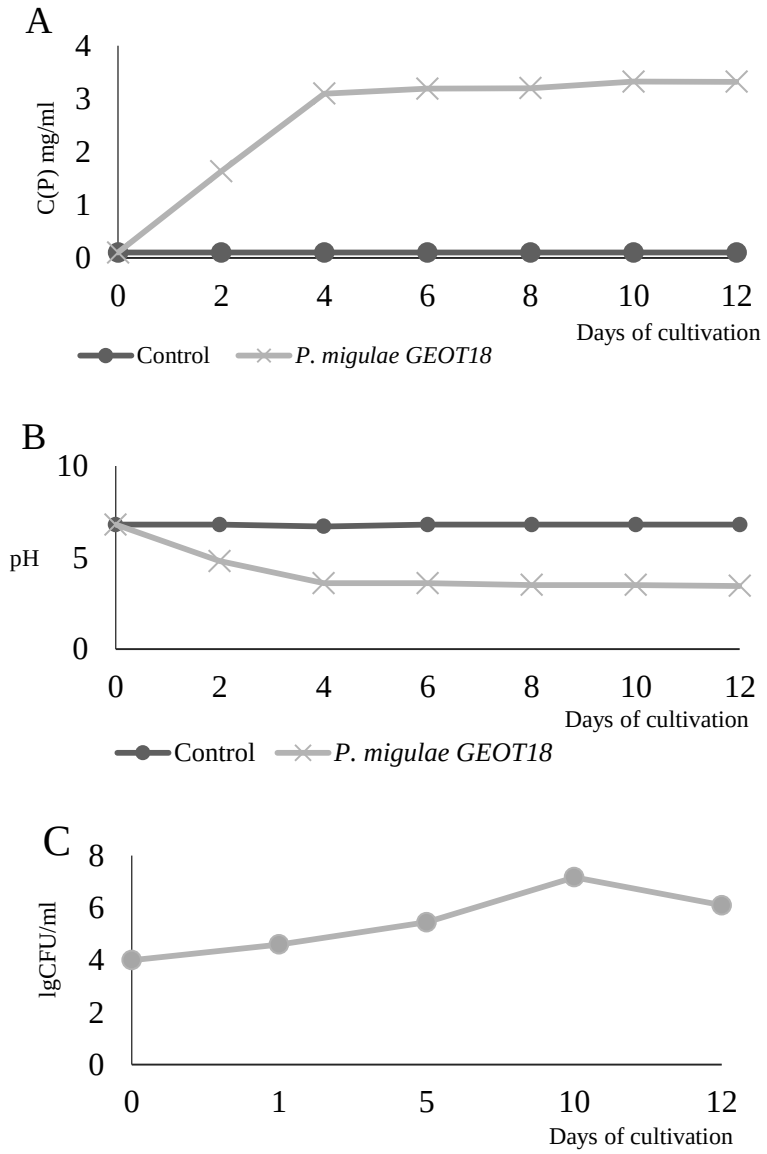


**Figure 2**. P-solubilizing activity of *Pseudomonas migulae* GEOT18: P content in the culture medium of *P. migulae* GEOT18 (A), Change in pH of medium of *P. migulae* GEOT18 (B), the dynamics of *P. migulae* GEOT18 culture (C)

**Table 1.** Effect of *P. migulae* GEOT18 on *Tagetes patula* growth

| Variant | Shoot length, cm | Root length, cm | Quantity of flowers | Root wet mass, g | Shoot wet mass, g | Wet mass of flowers, g | Root dry mass, g | Shoot dry mass, g | Dry mass of flowers, g |
|---|---|---|---|---|---|---|---|---|---|
| **NS** | 11,73 ± 1,43 | 3,36 ± 0,35 | 4,78 ± 0,49 | 0,10 ± 0,02 | 2,75 ± 0,40 | 0,13 ± 0,06 | 0,042 ± 0,008 | 0,29 ± 0,06 | 0,04 ± 0,03 |
| **NS-P** | 4,86 ± 0,61 | 3,86 ± 1,03 | 0,50 ± 0,23 | 0,06 ± 0,007 | 0,33 ± 0,03 | 0,006 ± 0,004 | 0,02 ± 0,003 | 0,03 ± 0,003 | 0,0005 ± 0,0002 |
| ***P. migulae* GEOT18 + NS-P** | 6,42 ± 0,53 | 3,85 ± 0,82 | 1.00 ± 0,36 | 0,06 ± 0,006 | 0,26 ± 0,03 | 0,03 ± 0,012 | 0,01 ± 0,001 | 0,50 ± 0,003 | 0,004 ± 0,002 |
| **TCP + NS-P** | 10,78 ± 1,09 | 3,11 ± 0,55 | 2,13 ± 0,35 | 0,10 ± 0,02 | 1,28 ± 0,20 | 0,21 ± 0,08 | 0,05 ± 0,01 | 0,13 ± 0,02 | 0,04 ± 0,01 |
| **TCP + *P. migulae* GEOT18 + NS-P** | 13,00 ± 3,10 | 4,64 ± 1,32 | 3,83 ± 0,30 | 0,09 ± 0,02 | 1,48 ± 0,39 | 0,48 ± 0,18 | 0,03 ± 0,007 | 0,17 ± 0,06 | 0,09 ± 0,04 |

NS –Nutrient solution
NS-P – Nutrient solution without phosphorus
TCP – tricalcium phosphate

The trial results showed that complete exclusion of phosphorus leads to a decrease in the height of sprouts of *T. patula* by 2.5 times, decreases the wet and dry mass of shoots by 8.5 times, the wet and dry mass of roots by 1.5 times, and the number of flower by 9.5 times. Wet and dry mass of flower decreased by 22 and 75 times, respectively, in the absence of phosphorus. These results indicate the necessity of phosphorus for the growth and development of generative organs. At the same time, it was revealed that the exclusion of phosphorus from the medium and introduction of the studied strain did not affect significantly the length of roots of *T. patula*.

Preliminarily introduction of TCP into the sand contributes to partial or complete restoration of these parameters. Thus, the height of sprouts, wet and dry root mass, and dry flower bud mass were completely restored. The number of flower and wet and dry mass of shoots restored partially.

It was established that the inoculation of plants with the strain in the trials with the preliminary introduction of TCP provided an increase in the height of shoots in comparison with the control by 20% (10.7 and 13 g in the control and trial, respectively) and dry mass of shoots by more than 30% in comparison with un-inoculated plants. The introduction of the *P. migulae* GEOT18 increases the number of flowers by 80% in comparison with un-inoculated plants. At the same time, the weight of flower increases by 2.2 times. The

influence of the studied strain on the development of the root system (weight and length) was not revealed.

The inoculation of plants with this strain in the trials without the preliminary introduction of TCP (in the lack of the source of phosphorus) increases the liner size of shoots (by 32% in comparison with the control), dry mass of shoots increased insignificantly (by 10%). Besides, there was an increase in the number of flower by 2 times in comparison with the un-inoculated control.

## Discussion

The application of microorganisms that mobilize phosphates is a good alternative to mineral phosphate fertilizers or additives, because they contribute to their better uptake. The *Pseudomonas* sp. GEOT18, isolated from the endosphere of *D. incarnata*, was identified by the method of molecular-genetic analysis that showed that this strain belongs to the genus *Pseudomonas* and show the sequence similarity 99% with *P. migulae*. The evaluation of phosphate solubilizing activity of the strain *in vitro* and uptake of insoluble TCP in the Pikovskaya agar medium showed that the content of free phosphate in the culture liquid was 3.25±0.05 mg/ml, which exceeds this parameter in the existing analogs (Oteino *et al*., 2015; Sharma *et al*., 2017; Suleman *et al*., 2018). During the cultivation of the *P. migulae* GEOT18 in the medium with TCP and phosphate mobilization, a decrease in the pH of cultural medium was observed from the initial value of 6.9 to 3.7. According to the published data, the acidification of the medium is provided by the expression of organic acids (acetic, citric, gluconic, and 2-ketogluconic acid) by the bacteria. The synthesis of these acids is one of the main mechanisms of insoluble soil phosphates mobilization by the bacteria (Khan *et al*., 2010). During the cultivation of *P. migulae* GEOT18, the maximum content of phosphates in the medium was observed in cases with the maximal number of bacterial cells and minimal pH in the cultural liquid, which agrees with the available published data (Li *et al*., 2019).

The majority of endophytic strains of bacteria have several properties that contribute to the growth and development of plants. One of the most significant properties is the synthesis of indole-3-acetic acid that controls a wide spectrum of the growth and development of plants: low concentratio2019ns of this acid stimulate the primary root elongation and high concentrations stimulate the formation of side roots and root hairs (Zhao, 2010; Saini *et al*., 2013). Besides, some strains can produce siderophores, low-molecular substances that chelate iron due to a high affinity to Fe (III). Thus, they increase its availability for plants, which is important in the conditions of iron deficiency in the soil.

Besides, the production of siderophores inhibits the development of pathogenic microflora because the formed iron chelates are not available for pathogenic microorganisms (Alexander *et al*., 1991). Biological nitrogen fixation is a process of reduction of molecular nitrogen to ammonium under the influence of nitrogenase observed in some endophytic microorganisms (Puri *et al*., 2018). The fixation of molecular nitrogen plays an important role in the mineral nutrition of plants because they cannot directly uptake molecular nitrogen.

The presence of these activities in the *P. migulae* GEOT18 indicated its high biotechnological potential. This strain is perspective for the production of biostimulators of plant growth.

The evaluation of the influence of the *T. patula* inoculation with this strain showed that *P. migulae* GEOT18 contributed to an increase in the vegetative mass (by more than 30%), mass of generative organs (by 2 times), and height of plants (by 30%). It was established that the inoculation of plants with the strain accelerates the formation of buds in plants by 2.5 weeks and prolongs flowering. The obtained data confirm that symbiotic bacteria *Pseudomonas* can increase the fertility of soil and the yield of plants. The lack of positive influence of the strain on the length and mass of roots can be explained by the synthesis of IAA at high doses that inhibit the development of the main root and stimulate the formation of root hairs. Besides, due to the availability of phosphorus, plants do not need to develop root system. The positive influence of the *P. migulae* GEOT18 on some morphometric parameters of the growth and development of plants in the trials without TCP can be explained by the presence of growth stimulating properties in the studied strain (synthesis IAA, nitrogen fixation, synthesis of siderophores). The obtained data indicate that the strain provides solubility and better uptake of nutrients, in particular, phosphorus, and can be considered as a perspective option for the production of phosphorus solubilizing biofertilizers.

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