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## Effect of seed bacterization with plant growth-promoting bacteria on wheat productivity and phosphorus mobility in the rhizosphere

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**Abstract:** Bacterization of the seeds of spring durum wheat with the strains of gram-positive aerobic spore-forming bacteria *Bacillus subtilis* IB-21 and *B. subtilis* IB-22 and gram-negative bacteria *Advenella kashmirensis* IB-K1 and *Pseudomonas extremaustralis* IB-K13-1A was performed to study its effect on the productivity of plants, their hormonal content and rhizosphere phosphorus (P) status in the field experiments. *A. kashmirensis* IB-K1 and *P. extremaustralis* IB-K13-1A were the most capable of mobilizing hardly soluble phosphates *in vitro*, while *P. extremaustralis* IB-K13-1A produced the greatest concentration of auxins. All the studied strains successfully colonized the plant root system, the level of colonization detected during the second leaf stage being the highest in the case of *A. kashmirensis* IB-K1 and *B. subtilis* IB-22. Seed treatment with all the tested bacterial species resulted in an increase in phosphate mobility in the rhizosphere. Auxin content in wheat roots was increased by bacterization of seeds with *P. extremaustralis* IB-K13-1 and *B. subtilis* IB-22. The maximum increase in components of wheat crop yield (the mass of grains in the main and axillary spikes) was detected during 3 vegetative periods (2016, 2017 and 2018) in the case of seed treatment with the strains inducing a significant increase in auxin content in the roots of the treated plants related to either the highest bacterial capacity of producing this hormone *in vitro* (in the case of *P. extremaustralis* IB-K13-1A) or root colonization (in the case of *B. subtilis* IB-22).

**Keywords:** spring wheat; plant hormones; phosphate-mobilizing bacteria; soil phosphate mobility

Acceleration of plant growth by the so-called plant growth promoting bacteria (PGPB) is related to their capacity to produce hormones, to increase the availability of mineral nutrients and water for plants and plant protection against diseases by PGPB (Marschner et al. 2011). These properties were detected in different strains of PGPB apart or in combination but were mostly considered independently from each other. Phosphate-containing minerals are hardly soluble complicating their uptake by plants, while microorganisms contribute to extracting soil phosphorus (P) by solubilization of phosphates.

Phosphate-mobilizing activity in bacteria is frequently accompanied by their capacity to produce auxins (Singh et al. 2014). Laboratory experiments previously showed that introduction of P-mobilizing and auxin-producing bacteria into the soil contributed to elevated content of auxins in the roots, increase in their mass and accumulation of phosphorus in the plants (Kudoyarova et al. 2017). However, direct introduction of bacteria into soil involves additional procedures like soil spraying, while after the pre-sowing seed treatment, bacteria are introduced into the soil together with the planted seeds.

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The goal of the present work was to study the capacity of phosphate-mobilizing and auxin-producing bacteria to colonize wheat rhizosphere under field conditions, to estimate the changes in hormone content in plants and the changes in phosphate status of the rhizosphere as well as the effects of seed bacterization on the components of crop yield.

## MATERIAL AND METHODS

**Bacterial strains and culture media.** Gram-positive aerobic spore-forming bacteria *Bacillus subtilis* IB-21 and *B. subtilis* IB-22 and gram-negative bacteria *Advenella kashmirensis* IB-K1 and *Pseudomonas extremaustralis* IB-K13-1A were obtained from the Laboratory of Applied Microbiology of the Ufa Institute of Biology, Ufa Federal Research Centre of the Russian Academy of Sciences. To study colonization of wheat root with bacteria, clones of *B. subtilis* IB-21, *B. subtilis* IB-22, *A. kashmirensis* IB-K1 resistant to 100 mg/mL rifampicin and a clone of *P. extremaustralis* IB-K13-1A resistant to 200 mg/mL streptomycin were obtained. Both *Bacillus* strains were cultivated in flasks with K1G medium (described in Arkhipova et al. 2005), while King B medium (King et al. 1954) was used for cultivation of bacteria of *P. extremaustralis* IB-K13-1A. *A. kashmirensis* IB-K1 was cultivated on modified K1G medium, where the stretch was substituted with glucose of the same concentration. Microorganisms were cultivated in a shaker (160 rpm) at 28°C for 48 h in the case of gram-negative and at 37°C for 96 h in the case of gram-positive strains. Microbe biomass was separated by centrifugation during 20 min at 4000 rpm and diluted in tap water to yield inoculate density of  $10^4$ – $10^7$  colony forming units (CFU) per seed. Carboxymethylcellulose sodium salt was added during bacterization and control treatment. Bacterial suspension with known titer was added into the flask with seeds and 4% carboxymethylcellulose sodium salt and shook for 10 min till adhesion of bacterial mass to the seeds, as judged by the absence of bacterial film on the flask walls.

Seeds from the control treatment variant were also placed in flasks with 4% carboxymethylcellulose sodium salt but without bacterium suspension and were shaken for 10 min.

Field experiments were carried out provided in 2016–2018. Spring wheat seeds (*Triticum durum* Desf., cv. Bashkirskaya 27) were sown at a depth of 5–6 cm. There were four 1.5 m<sup>2</sup> plots – replicates – in an ex-

perimental field (54°50'N, 55°44'E, 170 m a.s.l.). The soil was a leached Chernozem of South wooden steppes of Bashkortostan. The ploughing horizon of unfertilized soil contained 3.65% C<sub>org</sub> (organic carbon), was characterized by slightly acidic soil solution, high content of absorbed bases with dominating calcium (350 mmol/kg of Ca<sup>2+</sup>, 120 mmol/kg of Mg<sup>2+</sup>) and moderate availability of mobile phosphorus and alkaline hydrolysable nitrogen. Crop yields were measured in 2016, 2017 and 2018; seasons differed in the amount of rainfall – about 100 mm in 2016 and 2018 and about 280 mm in 2017 in April–June. The average temperature in May–June was the lowest in 2018 (12°C), highest in 2016 (17°C) and intermediate in 2017 (14°C).

Plants were sampled four times for measuring the number of bacteria colonizing plant root system. For a hormone assay, plants were sampled at the stage of the second leaf. At the end of vegetation, the content of mobile phosphorus was analysed, as well as crop yield components.

Solubilization of mineral and organic phosphates was measured as the diameter of the clearance zone and the area of clearance zone was calculated of corresponding substances in the agar medium (Pikovskiy 1948).

Number (CFU/seed or g rhizosphere soil) of introduced bacteria was estimated in the seeds and plant rhizosphere. Seeds were slightly homogenized in a mortar with sterile tap water. Roots we detached from the plants and after shaking off the non-rhizosphere soil, they were moistened with sterile tap water, placed into a mortar and slightly homogenized. Flasks with roots and rhizosphere soil were shaken for 20 min. The obtained dilutions of suspension were sown on the nutrient agar plates with corresponding antibiotics (see above) for selecting resistant bacteria.

Hormones were extracted with 80% ethanol from shoots and roots of 5 plants (in one replicate) as described by Yurkov et al. (2017). The ethanol extract was separated by centrifugation and evaporated. Extraction of abscisic acid (ABA) and indoleacetic acid (IAA) from aliquots of aqueous residues and from bacterial culture media was performed with the diethyl ether according to the modified method as described by Kudoyarova et al. (2017). Cytokinins (zeatin, its riboside, N-glucoside and nucleotide) were concentrated on C-18 column and separated with the TLC chromatography as described by Kudoyarova et al. (2014). Hormones were immunoassayed using the corresponding specific antibodies.

P intensity (indicator of P concentration in the soil solution directly available for the plant) and P

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Table 1. Phosphate mobilization ability ( $\text{mm}^{2*}$ ) and auxin production of clones resistant to antibiotics and used for seed bacterization of spring wheat

Characteristic	<i>Advenella kashmirensis</i> <sup>rif</sup>	<i>Pseudomonas extremaustralis</i> <sup>str</sup>	<i>Bacillus subtilis</i> <sup>rif</sup>	<i>B. subtilis</i> <sup>rif</sup>
	IB-K1	IB-K13-1A	IB-21	IB-22
$\text{Ca}_3(\text{PO}_4)_2$	700 ± 56	490 ± 75	14 ± 2	40 ± 7
$\text{C}_6\text{H}_{18}\text{O}_{24}\text{P}_6$	345 ± 43	490 ± 47	16 ± 5	44 ± 7
Auxin production (ng/mL)	13 ± 1	880 ± 92	313 ± 35	60 ± 12

Values are means ± standard error. \*We measured diameter as indicated in the Material and Methods section and calculated the area of the clearance zone

capacity of the soil (content of solid phase phosphate compounds that may be released in the long term providing the level of P intensity in the soil solution) were determined based on the strength of the extraction method (Dalal and Hallsworth 1976) before sowing and at the crop maturity stage. For determination of P capacity factor, soluble phosphates were extracted from the soil with 0.5 mol/L solution of acetic acid (pH 2.5; w/v 1:25) as described by Kudoyarova et al. (2017). P intensity was measured by extracting phosphates with 0.015 mol/L  $\text{K}_2\text{SO}_4$  (w/v 1:5). Determination of phosphates in soil extract was carried out with the molybdenum-blue method using stannous chloride as the reducing agent (Allen et al. 1974).

**Statistical analysis.** Means ± standard error present in the tables and figures were calculated using MS Excel (Microsoft, USA). A number of replicates was different with each characteristic and indicated in the illustrations. Significance of the differences between means was estimated by the ANOVA (*LSD* (least significant difference) test) and *t*-test.

## RESULTS AND DISCUSSIONS

Table 1 shows different capacity of the studied bacterial strains to produce auxins. The highest concentration of IAA was detected in the culture media of *P. extremaustralis* IB-K13-1A. Solubilization of phosphates was more actively performed by the gram-negative bacteria (*A. kashmirensis* IB-K1 and *P. extremaustralis* IB-K13-1A). It was important to follow how these characteristics manifested *in vitro* influenced soil P mobility and plant hormone content and productivity. The latter effects were likely to depend on the capacity of bacteria to colonize plant root system.

Screening of strains with a high rate of rhizosphere colonization is a major challenge for PGPB commercialization (Backer et al. 2018). Figure 1 shows that all the studied strains successfully colonized

the root system. Both *Bacillus* strains have been recovered from the wheat rhizosphere up to the crop harvesting. *B. subtilis* IB-22 had the highest density of cell application on the seeds, and its presence in the rhizosphere remained high at the stage of the second leaf. A maximum number of bacteria of *A. kashmirensis* IB-K1 and *P. extremaustralis* IB-K13-1A in the root system of wheat was at the stage of the second leaf and tillering correspondingly. A number of bacteria of *B. subtilis* IB-21 remained mostly lower than in the case of other strains. Spore-producing bacteria of *Bacillus* genus successfully colonized wheat rhizosphere, when applied at  $10^4$ – $10^6$  CFU per seed (Kuzmina and Melentiev 2003). *A. kashmirensis* was shown to be present in the rhizosphere of wild plants (Poroshina et al. 2015). The novelty of our results is in showing their capacity to colonize wheat rhizosphere through seed inoculation. This study was

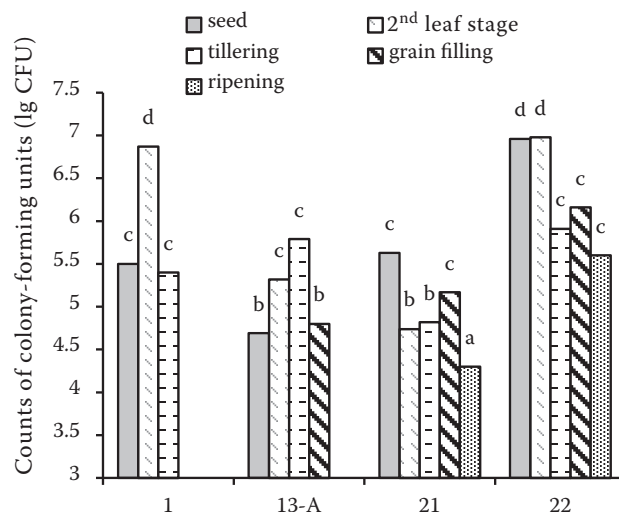


Figure 1. Counts of colony-forming units (CFU) of introduced bacteria in the seeds and wheat root system: 1 – *Advenella kashmirensis* IB-K1; 13-1A – *Pseudomonas extremaustralis* IB-K13-1A; 21 – *Bacillus subtilis* IB-21; 22 – *B. subtilis* IB-22. Significantly different means for CFU counts are labelled with different letters ( $P < 0.05$ , *LSD* (least significant difference) test, ANOVA)

the first to show the capacity of *P. extremaustralis* to colonize wheat root rhizosphere and to persist in it for a sufficiently long time to enable the effects on plant development and soil processes.

Many rhizosphere bacteria can synthesize auxins *in vitro* (Spaepen and Vanderleyden 2011). Since plants are capable of auxin uptake from the nutrient medium (Sussman and Goldsmith 1981), inoculation of auxin-producing bacteria is likely to influence IAA content in the plants. Nevertheless, bacterial effects on the concentration of hormones in plants should be confirmed by direct measurements. Figure 2a shows that the concentration of IAA was higher than in control in the roots and shoots of wheat plants inoculated with the cells of *P. extremaustralis* IB-K13-1A and *B. subtilis* IB-22. Although *B. subtilis* IB-22 produced fewer auxins *in vitro*, this was likely to be compensated by the high level of their root colonization. *B. subtilis* IB-21 did not increase root auxins over the control despite rather a high capacity to produce these hormones *in vitro* that was likely to be due to their low capacity to colonize

the roots (Figure 1). Thus, the effect of bacteria on plant hormonal status is likely to depend on both factors: the ability of bacterial strains to produce hormones and efficiency of their root colonization. These results may explain the discrepancy between auxin production by PGPB *in vitro* and their effect on the IAA content in the inoculated plants.

The difference in ABA level was evident in the roots in the case of plants inoculated with the cells of *B. subtilis* IB-22 strain and in the shoots of plants inoculated with *A. kashmirensis* IB-K1 as compared to the control (Figure 2b). Figure 2c shows that only the treatment with the suspension of *B. subtilis* IB-22 bacteria resulted in about a 2-fold increase in the level of cytokinins in the roots.

At the stage of the second leaf, root dry mass was increased by the inoculation of seeds with the suspensions of *A. kashmirensis* IB-K1, *P. extremaustralis* IB-K13-1A and *B. subtilis* IB-21 cells.

Soil microorganisms are capable of effective solubilization of phosphates by excreting organic acids and phosphatases (Richardson et al. 2009 and ref-

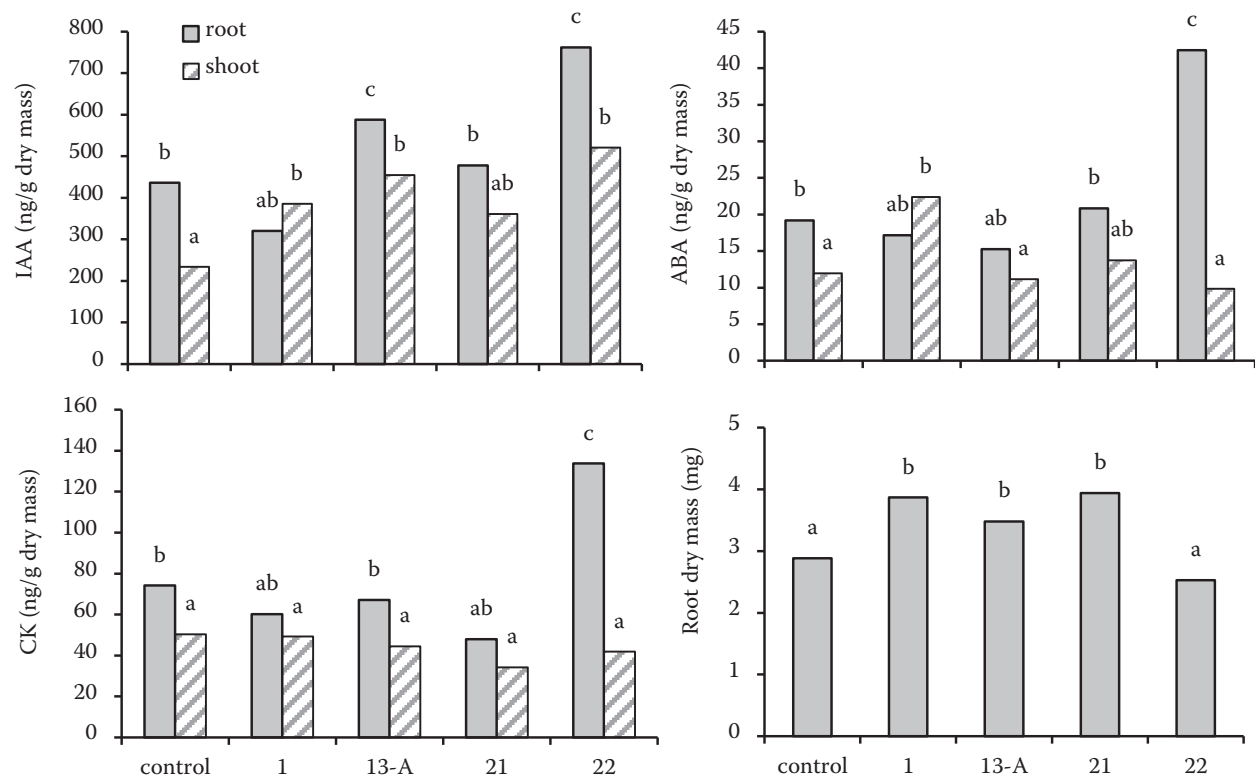


Figure 2. Concentration of indoleacetic acid (IAA); abscisic acid (ABA), and cytokinins (CK) in shoots and roots and root dry mass of spring wheat plants at the stage of the second leaf after seed bacterization 1 – *Advenella kashmirensis* IB-K1; 13-A – *Pseudomonas extremaustralis* IB-K13-A; 21 – *Bacillus subtilis* IB-21; 22 – *B. subtilis* IB-22. Significantly different means for each index are labelled with different letters ( $n = 4$  for hormone assay and  $n = 20$  for root dry mass measurements,  $P < 0.05$ , LSD (least significant difference) test, ANOVA)

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Table 2. Effect of spring wheat seed bacterization on the soil phosphorus (P) status (P capacity and intensity measured during extraction with acetic acid and potassium sulfate, correspondingly) before sowing (BS) and at the crop maturity stage (MS)

Variant	P capacity (mg/kg)		P intensity (mg/L)	
	BS	MS	BS	MS
Control	96.4 ± 3.1 <sup>b</sup>	80.4 ± 2.7 <sup>a</sup>	0.18 ± 0.01 <sup>a</sup>	0.18 ± 0.01 <sup>a</sup>
<i>Advenella kashmirensis</i> IB-K1	95.7 ± 3.0 <sup>b</sup>	94.8 ± 3.1 <sup>b</sup>	0.17 ± 0.02 <sup>a</sup>	0.24 ± 0.02 <sup>b</sup>
<i>Pseudomonas extremaustralis</i> IB-K13-1A	96.3 ± 3.1 <sup>b</sup>	93.6 ± 3.0 <sup>b</sup>	0.18 ± 0.01 <sup>a</sup>	0.23 ± 0.01 <sup>b</sup>
<i>Bacillus subtilis</i> IB-21	95.9 ± 2.9 <sup>b</sup>	93.9 ± 2.9 <sup>b</sup>	0.18 ± 0.01 <sup>a</sup>	0.21 ± 0.01 <sup>b</sup>
<i>B. subtilis</i> IB-22	96.7 ± 3.2 <sup>b</sup>	95.1 ± 3.1 <sup>b</sup>	0.18 ± 0.02 <sup>a</sup>	0.21 ± 0.02 <sup>b</sup>

Values are means ± standard error ( $n = 7$ ). Significantly different means for P capacity and intensity are labelled with different letters ( $P \leq 0.05$ , LSD (least significant difference) test, ANOVA)

erences therein; Whitelaw et al. 1999). The content of available P usually decreased by the end of the vegetation period. In the control (in the soil with non-inoculated plants), P capacity was lower at the stage of full maturity than before sowing, while the decline in soil P capacity was insignificant in all of the variants of inoculation (Table 2) suggesting increased P availability by bacteria. Activated mobilization of phosphates by bacteria is supported by 17–33% increase in the extent of phosphate mobility (P intensity determined during soil extraction with potassium sulfate) as compared to the control and initial values (before sowing).

*A. kashmirensis* IB-K1 and *P. extremaustralis* IB-K13-1A showed high phosphate solubilizing capacity *in vitro* (Table 1) and increased phosphorus mobility in the soil, which is likely to be due to the effect of these bacteria directly on the soil phosphates. Unlike *A. kashmirensis* IB-K1 and *P. extremaustralis* IB-K13-1A, *B. subtilis* IB-21 and *B. subtilis* IB-22 showed a far lower capacity to mobilize phosphates *in vitro*. Nevertheless, the extent of phosphorus mobility in soil with plants treated with these bacteria was

greater than in control. Plant hormones are capable to increase root exudation (Wittenmayer and Merbach 2005, Kudoyarova et al. 2014) and strains of *B. subtilis* IB-21 and *B. subtilis* IB-22 were capable of producing hormones (*B. subtilis* IB-21 – auxins, and *B. subtilis* IB-22 – cytokinins (Arkhipova et al. 2005). The results suggest that increased phosphate mobility in the soil may be due to the possible stimulation of root exudation due to bacteria-produced hormones.

The 2017 year was the most favourable for crop production supported by the highest values of indicators of wheat productivity (Table 3). This was likely to be due to the level of rainfalls and temperature being closer to optimal in 2017 than during other vegetation seasons. Wheat crop yield was the lowest in 2018 seemingly due to cool temperature at the beginning of the vegetation season. Despite the difference in absolute levels of wheat productivity, seed inoculation increased crop yield detected in all the three vegetation seasons. Still, the effect of inoculation with *P. extremaustralis* IB-K13-1A and *B. subtilis* IB-22 on the grain mass of the main spike was greater during the least favourable year (2018), when this index in

Table 3. Analysis of the effect of spring wheat seeds inoculation on the components of the crop yield ( $n = 120$ )

Mass of grains	2016					2017				2018			
	control	IB-K1	IB-K13-1A	IB-21	IB-22	control	IB-K1	IB-K13-1A	IB-22	control	IB-K1	IB-K13-1A	IB-22
Main spike (g)	1.45 <sup>a</sup>	1.45 <sup>a</sup>	1.64 <sup>b</sup>	1.56 <sup>b</sup>	1.58 <sup>b</sup>	1.97 <sup>a</sup>	1.99 <sup>a</sup>	2.18 <sup>b</sup>	2.16 <sup>b</sup>	0.80 <sup>a</sup>	0.90 <sup>a</sup>	1.07 <sup>b</sup>	1.02 <sup>b</sup>
Axillary spikes per plant (g)	0.61 <sup>a</sup>	0.82 <sup>a</sup>	1.16 <sup>b</sup>	1.04 <sup>b</sup>	1.19 <sup>b</sup>	0.82 <sup>a</sup>	0.87 <sup>a</sup>	1.51 <sup>b</sup>	1.59 <sup>b</sup>	0.37 <sup>a</sup>	0.48 <sup>a</sup>	0.53 <sup>b</sup>	0.50 <sup>b</sup>

IB-K1 – *Advenella kashmirensis* IB-K1; IB-K13-1A – *Pseudomonas extremaustralis* IB-K13-1A; IB-21 – *Bacillus subtilis* IB-21; IB-22 – *B. subtilis* IB-22. Significantly different means for each index are labelled with different letters ( $P \leq 0.05$ , LSD (least significant difference) test, ANOVA)

control was about 1.8 times lower than in 2016. The grain mass of the main spike was 30% heavier in the case of inoculation with *P. extremaustralis* IB-K13-1A and *B. subtilis* IB-22 in 2018 and only about 10% heavier in 2016 and 2017. Increment of a grain mass of the main spike was significantly higher in 2018 than in 2016 and 2017 ( $P \leq 0.05$ , *t*-test). Bacterial treatment with *P. extremaustralis* IB-K13-1A and *B. subtilis* IB-22 increased the weight of grains in axillary spikes to a greater extent than those of the main spikes in 2016 and 2017 (almost 2-fold increase in 2016 and 2017 against not more than 40% increase in 2018, the difference being significant at  $P \leq 0.05$ , *t*-test). Our data showing greater positive effects of bacterization on wheat productivity during the least favourable year (2018) are in agreement with earlier reports showing a higher efficiency of PGPB under stressful conditions (Kuzmina and Melentev 2003, Rubin et al. 2017). The capacity of cytokinins to influence shoots branching is well-known to bring about increased spike and grain number (Wilkinson et al. 2012). This information allows attributing the effect of *B. subtilis* IB-22 on the number of grains in the spikes to its capacity to produce cytokinins (Arkhipova et al. 2005).

*Azospirillum brasilense* inoculation was shown to improve *Arabidopsis thaliana* seed yield in correlation with incremented ABA levels (Cohen et al. 2015). In our experiments, no such correlation was detected, since although ABA content in roots was elevated by inoculation of *B. subtilis* IB-22 strain resulting in higher wheat productivity, *P. extremaustralis* IB-K13-1A did not influence the ABA content in the plants despite a positive effect of the strain on the grain mass produced by inoculated plants (Figure 2b, Table 3), while *A. kashmirensis* IB-K1 increased the ABA concentration in wheat shoots, but did not influence plant productivity.

It is of interest that *A. kashmirensis* IB-K1 failed to influence most of the components of crop yield despite their capacity to solubilize phosphates (Table 3). Meanwhile, high capacity to influence plant productivity was detected for *P. extremaustralis* IB-K13-1A, whose high capacity to mobilize phosphates was accompanied by auxin production *in vitro* and a significant effect on root auxin content. The results suggest that the capacity of bacteria to mobilize phosphates is not the only factor responsible for increasing plant productivity.

Our experiments were performed in only one location; thus, additional experiments are needed to test

the applicability of the revealed regularity to other locations. Nevertheless, this study has shown that bacteria introduced in the wheat rhizosphere by their application of the seeds, may serve as perspective agents for mobilizing phosphates and increasing plants productivity. The maximum productivity was achieved in the variants with *P. extremaustralis* IB-K13-1A and *B. subtilis* IB-22 seed treatment, as related to their high capacity to mobilize phosphates of the soil and to produce plant hormones and influence hormone content in inoculated plants.

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