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Bacteria associated with grapevine (*Vitis vinifera* L.) rhizosphere and their efficacy in plant growth promotion

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Abstract

Plant growth, development and stress resistance depend on the presence of plant growth-promoting rhizobacteria (PGPR) in its rhizosphere. The detection of the highly active PGPR is of high importance due to their possible application as microbial inoculants for plant growth promotion (PGP) in agriculture. In this study, we report on PGPR from the rhizosphere of grapevine (*Vitis vinifera* L.) growing in Uzbekistan, as it was not studied before. Based on the screening of 37 isolates from grapevine rhizosphere for stimulation of wheat seed germination, just two isolates, BDI-1 and BDI-2 were chosen as the most active. In laboratory conditions, the isolates BDI-1 and BDI-2 increased wheat root length up to 1.48 and 1.5 times and shoot length up to 1.59 and 1.64 times, respectively, as compared to the control. Based on 16S rRNA gene analysis and comparison with the relative strains registered in GenBank of the National Center for Biotechnology Information (NCBI), the isolates BDI-1 and BDI-2 were identified as *Pantoea agglomerans* and *Priestia megaterium,* accordingly. Their 16S rRNA gene nucleotide sequences were deposited to GenBank under the accession numbers OP727725 for BDI-1 and OP782582 for BDI-2. Both isolates were phenotypically characterized and demonstrated phosphate-solubilizing and nitrogen-fixing abilities, producing indole-3-acetic acid (IAA) in a high amount; however, BDI-1 also produced siderophores and BDI-2-1-aminocyclopropane-1-carboxylate (ACC) - deaminase. Due to these features, the bacteria showed their high activity in the promotion of plant growth and seed germination. In conclusion, according to our results, *P. agglomerans* BDI-1 and *P. megaterium* BDI-2 are promising PGPR, which can be applied as microbial inoculants for plant growth improvement.

Keywords

Pantoea agglomerans; PGPR; *Priestia megaterium*; rhizosphere; *Vitis vinifera* L.; grapevine

Introduction

Vitis vinifera L., or grapevine, is a widely cultivated woody fruit crop whose annual grape production was 76,750,674 tons in 2022 (1). It was domesticated from its wild ancestor, *Vitis vinifera* subsp. sylvestris (Willd.) Hegi, naturally occurring from southwestern Asia and Mediterranean Europe (2). The growth and productivity of

a grapevine as well as of other plants depends on the availability of nutrients in the soil. However, the majority of nutrients in soil is in stable forms and cannot be consumed directly by plant roots. It is well known that microbial communities living in the rhizosphere, or inside of plants, play an important role in supporting plant growth and development in different soils and climatic zones using different mechanisms (3, 4). Plant growth-promoting rhizobacteria (PGPR) can make insoluble minerals containing phosphorus and potassium available to plants by solubilizing those using phosphatases and some organic acids like hydrogen cyanide (5, 6). Some bacteria can supply plants with available nitrogen using a special nitrogenase enzymatic complex, which converts atmospheric nitrogen to ammonium (NH_4^+) (7). PGPR can also produce phytohormones, such as auxin indole-3-acetic acid (IAA), improving root growth by elongation and branching, letting the plant root system cover more area and uptake more water and nutrients (8, 9). Some bacteria can contribute to plant growth by producing siderophores, which can bind iron and make it unavailable for some plant pathogenic fungi, thus protecting plants from diseases (10, 11). Other PGPR alleviates plant stress by cleaving an ethylene (stress hormone)precursor-1-aminocyclopropane-1-carboxylic acid with 1-aminocyclopropane-1-carboxylate (ACC) - deaminase (12). There were several reports about PGPR isolated from the rhizosphere of different crops such as soybean (*Glycine max*) and chilli (*Capsicum annuum*) in India (13), maize (*Zea mays*) in Africa (14), one report about grapevine (*Vitis vinifera*) in Turkey (15) and others crops. However, the microbial communities of PGPR vary in the rhizosphere of the same plant species grown in different geographical zones with specific climate conditions and soil types (16). Up to now, there were no reports about PGPR from the rhizosphere of a grapevine grown in Uzbekistan with its specific soils and climate.

Our aims were: 1) isolate the rhizosphere bacteria contributing to grapevine growth and fitness in local soils of Uzbekistan; 2) study some plant growth promoting (PGP) and phenotypic characteristics of the chosen bacteria.

Materials and Methods

Samples isolation

The light serozyom soil samples in the amount of 10 were collected from the rhizosphere of a grapevine (*Vitis vinifera* L.) growing in the Samarqand region of the Kattakorgan district of Uzbekistan. The root system with the stuck soil particles was transferred into sterile plastic bags and brought to the laboratory for further analysis.

Bacteria isolation

The bacteria were isolated from the collected grapevine rhizosphere soil as follows: 10 g of the collected grapevine roots with rhizosphere soil was mixed thoroughly with 100 mL of sterile water and serially diluted up to 1×10^{-4} and 1×10^{-5}. 100 µL of final soil suspension was transferred and spread with a triangle onto the surface of Petri dishes with peptone agar. The dishes were left at 28°C for incubation and after 48 h, they were checked for the presence of bacterial colonies. The grown bacterial colonies, different in shape, size and color were isolated by transfer to individual Petri dishes with peptone agar and purified.

Screening for bacterial activity in the stimulation of seed germination

The isolated bacteria were tested in the stimulation of wheat (*Triticum aestivum* L.) seed germination. The isolated pure bacterial cultures were used as inoculants for wheat seeds to check their efficiency in stimulating seed germination. The bacteria were cultivated separately in nutrient broth at 28 °C for 72 h and the concentration of cells was adjusted to 10^7 CFU/mL. The seeds of wheat were externally sterilized by keeping in 2.5% NaClO for 3 min and rinsed in sterilized tap water (17). The outer sterilized seeds (100) were inoculated with each bacterial isolate by keeping them in bacterial suspension for 30 min and put into sterile Petri dishes with moist filter paper. As a control, the nutrient broth medium was used. The dishes were left in a dark place and the day-night temperature was 28-18 °C. The germination rate of seeds was recorded four times: after 12, 24, 36 and 48 h as a number of germinated seeds (from 100 seeds).

Screening of bacteria for plant growth-promoting properties

The bacteria were cultivated separately in nutrient broth at 28°C for 72 h and the concentration of cells was adjusted to 10^7 CFU/mL. The seeds of wheat were externally sterilized by keeping in 2.5 % NaClO for 3 min and rinsed in sterilized tap water (17). One hundred sterilized seeds were inoculated with each bacterial isolate by keeping them in bacterial suspension for 30 min and transferred into wide plastic pots with sterile light serozyom soil. The pots were marked and set up in random places inside the plant growth chamber. We used pots in three replications and there were 10 seeds inoculated with the same bacterial isolate inside each pot. The plants were grown at 30 °C in the daytime and 20 °C at night and watered as required. The shoots and roots length were measured 10 days after seeds sowing.

Bacteria identification

The bacteria were identified using 16S rRNA gene analysis. To amplify the 16S rRNA region of the samples, we used a pair of primers BAK11w 5'-AGTTTGATCMTGGCTCAG-3' and BAK2 5'-GGACTACHAGGGTATCTAAT-3', which are optimal for identifying a wide range of bacterial DNA (18). The amplification reaction was carried out using an iCycler thermal cycler (Bio-Rad Laboratories, USA), programmed for the following reaction conditions: preliminary denaturation 96°C-1 min, 30 cycles, including 96°C-10 sec, 55°C-20 sec, 72°C -30 sec and final elongation 55°C-20 sec, 72°C-3 min. The volume of the reaction mixture was 25 µL, containing 0.3 mM of primers BAK11w and BAK2, 0.2 mM of each nucleotide, 2.5 µL of 10X Encyclo buffer, 0.5 µL of 50X Encyclo polymerase and 50 ng of DNA sample. The PCR products were analyzed in 1% agarose gel with the addition of ethidium bromide. The resulting PCR product was cut from the gel, purified and used in the sequencing reaction using the BigDye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) according to the manufacturer's recommendations (Thermo Fisher Scientific, USA). The 16S rRNA gene sequences were compared with the identical sequences of the GenBank (NCBI) (http://www.ncbi.nlm.nih.gov/).

Study of the taxa evolutionary relationships

The neighbor-joining method was used to infer the evolutionary history (19). The associated taxa clustered together in the bootstrap test (500 replicates), according to Felsenstein (20). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The Maximum Composite Likelihood method (21) was utilized to compute the evolutionary distances. The evolutionary analyses were conducted in MEGA X (22).

Study of bacteria PGP properties

The N_2-fixation of bacteria were checked by the ability to grow on malate nitrogen-deficient medium (g/L): sodium malate - 5, K_2HPO_4 - 0.5, KH_2PO_4 - 0.4, $MgSO_4 \cdot 7H_2O$ - 0.2, NaCl - 0.1, $CaCl_2$ - 0.02, $FeCl_3$ - 0.01, $Na_2MoO_4 \cdot 2H_2O$ - 0.002, agar - 15, pH 7.2-7.4, modified with yeast extract - 50 mg/L (23). Sarwar and Kremer's method (24) was used to check the production of IAA. The bacterial suspension was stabilized at 1×10^8 CFU/mL and poured into tubes containing 10 % tryptic soy agar (25) modified with L-tryptophan -5 mmol/L. After 24 h of cultivation at 28 °C, the suspension was centrifuged for 15 min at 8000×g and the supernatant was gathered. The Salkowski reagent (mixture of 7.9 mol/L H_2SO_4 and 0.5 mol/L $FeCl_3$) was mixed with the supernatant in a ratio of 1:1 (v/v) and stored for 30 min at room temperature in the dark. The presence of pink color indicated the production of IAA, which was measured with a spectrophotometer at 530 nm. The standard curve was drawn using different concentrations of IAA solutions. The phosphate solubilizing ability was tested by the cultivation of bacteria on NBRIP medium (%): glucose - 1, $MgCl_2$ - 0.5, $Ca_3(PO_4)_2$ - 0.5, $MgSO_4 \cdot 7H_2O$ - 0.025, KCl - 0.02, $(NH_4)_2SO_4$ - 0.01 and agar - 1.5. The formation of colonies after bacteria incubation at 28°C for 96 days indicated the ability to use $Ca_3(PO_4)_2$ as a sole phosphate source (26). The ACC - deaminase production was based on the ability of the tested bacteria to utilize 1-aminocyclopropane-1-carboxylate as a source of nitrogen. The basal medium, modified with ACC 3.0 mM was utilized for bacterial cultivation. As a positive control, we added $(NH_4)_2SO_4$ into media and didn't add any source of nitrogen for the negative (27). The siderophores-producing ability was tested by cultivating the bacteria on chrome azurol sulphonate agar (CAS) at 28 °C for 96 h. The colonies surrounded by an orange color zone were positive in siderophores production (28).

Phenotypic characterization of bacteria

The biochemical, morphological and physiological characteristics of bacteria isolates were identified based on the standardized methods recommended in Bergey's Manual of Systematic Bacteriology (29).

Statistical analysis

The analysis of variance in the Microsoft Excel 2010 package was utilized to test the data's statistical significance. The results of the plant growth experiment were subjected to analysis of variance (ANOVA) with SPSS software (v. 15) at $p \leq 0.05$. The least significant difference test ($p \leq 0.05$) was used for conducting mean comparisons. The results are presented as average means and standard error. All tests for seed germination, plant growth-promoting and phenotypic characteristics of bacteria were conducted minimum in three replications.

Results

Screening of bacteria for wheat seed germination

In total, 37 isolates different in their colonies color, size, density and shapes were isolated from the soil samples of a grapevine rhizosphere. The isolates were checked and screened for their activity in the stimulation of wheat seed germination in Petri dishes after 12, 24, 36 and 48 h (Fig. 1).

As you can see from Fig. 1, there were no germinated seeds in control, BDI-6, BDI-10, BDI-21 and BDI-30 after 12 h. However, at the same time, the germination rate after seed inoculation with bacterial isolates BDI-1, BDI-2, BDI-8, BDI-14, BDI-16, BDI-27 and BDI-37 was 7, 8, 6, 5, 5, 5 and 6 % consecutively. In 24 h, the same isolates BDI-1, BDI-2, BDI-8, BDI-14, BDI-16, BDI-27 and BDI-37 showed the highest seed germination rate: 38, 41, 34, 36, 33, 34 and 36 % consecutively in comparison with other bacterial isolates and control. The most interesting results were after 36 h when the seed germination rate increased up to 91 and 94 % in BDI-1 and BDI

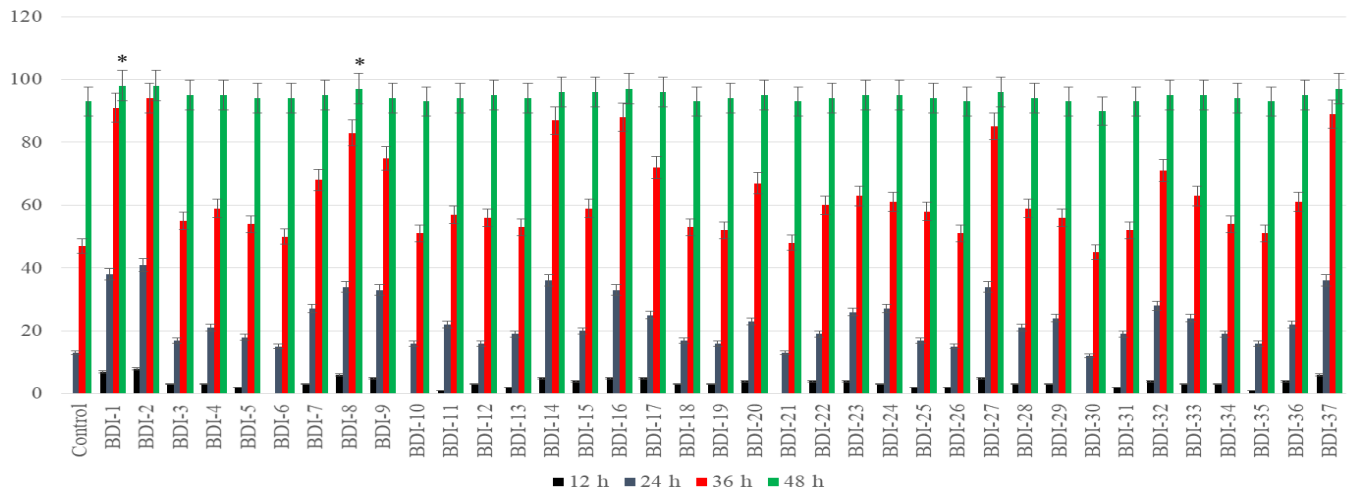

Fig. 1. The influence of wheat seeds inoculation with isolated rhizosphere bacteria on germination rate (%) in 12, 24, 36 and 48 h.

*- statistically significant at $p \leq 0.05$

-2 accordingly and that was 1.9 and 2 times more than in the control. The other isolates showed results less than 90 % of seed germination rate. The isolates BDI-1 and BDI-2 showed the highest results in seed germination (98 %) after 48 h.

Screening of bacterial isolates for wheat growth promotion

The efficacy of the isolated rhizosphere bacteria in wheat growth stimulation was tested in a pot experiment (Fig. 2). The majority of the isolates improved plant growth after seed inoculation. The isolates BDI-8, BDI-14, BDI-16, BDI-27 and BDI-37 increased root length up to 1.38, 1.36, 1.36, 1.33 and 1.4 times consecutively in comparison with the control. However, the best activity demonstrated isolates BDI-1 and BDI-2, which increased root length up to 1.48 and 1.5 times and shoot length up to 1.59 and 1.64 times, respectively, as compared to the control.

Bacteria identification

The identification of the most active isolates, BDI-1 and BDI-2 was based on their 16S rRNA gene comparison with genes of relative strains registered in GenBank of NCBI (Table 1). According to 16S rRNA gene analysis, the isolate BDI-1 is related to *P. agglomerans.* It was deposited to GenBank under accession number OP727725 and called *P. agglomerans* BDI-1. The isolate BDI-2 was related to *P. megaterium* and deposited to GenBank under accession number OP782582. The phylogenetic trees of the strains *P. agglomerans* BDI-1 and *P. megaterium* BDI-2 and their closest relative strains from GenBank were constructed in MEGA X software using the neighbor-joining method (Fig. 3 and 4).

Plant growth-promoting characteristics of bacteria

The plant growth-promoting characteristics of the strains *P. agglomerans* BDI-1 and *P. megaterium* BDI-2 were studied (Table 2). Both strains showed phosphate-solubilizing and nitrogen-fixing abilities that produced IAA in high amounts. *P. megaterium* BDI-2 also produced ACC - deaminase and

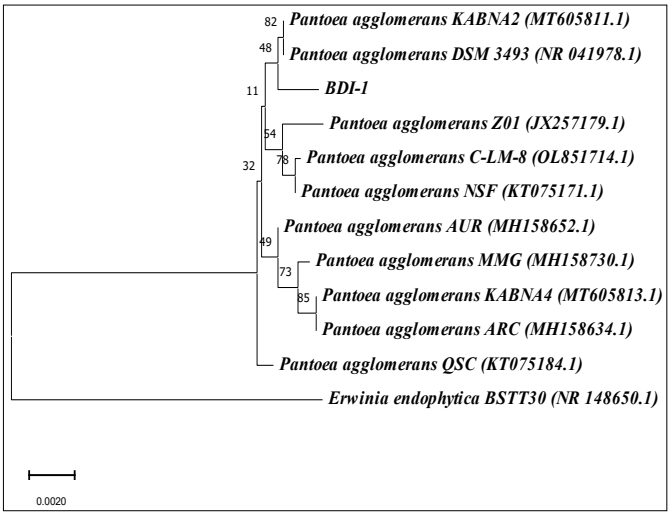

Fig. 3. The phylogenetic tree of the strain *Pantoea agglomerans* BDI-1 with the relative strains registered in GenBank (NCBI) based on 16S rRNA gene comparison.

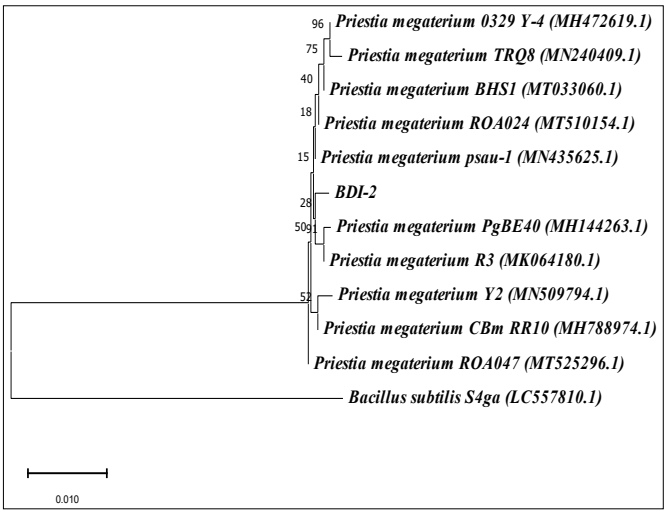

Fig. 4. The phylogenetic tree of the strain *Priestia megaterium* BDI-2 with the relative strains registered in GenBank (NCBI) based on 16S rRNA gene comparison.

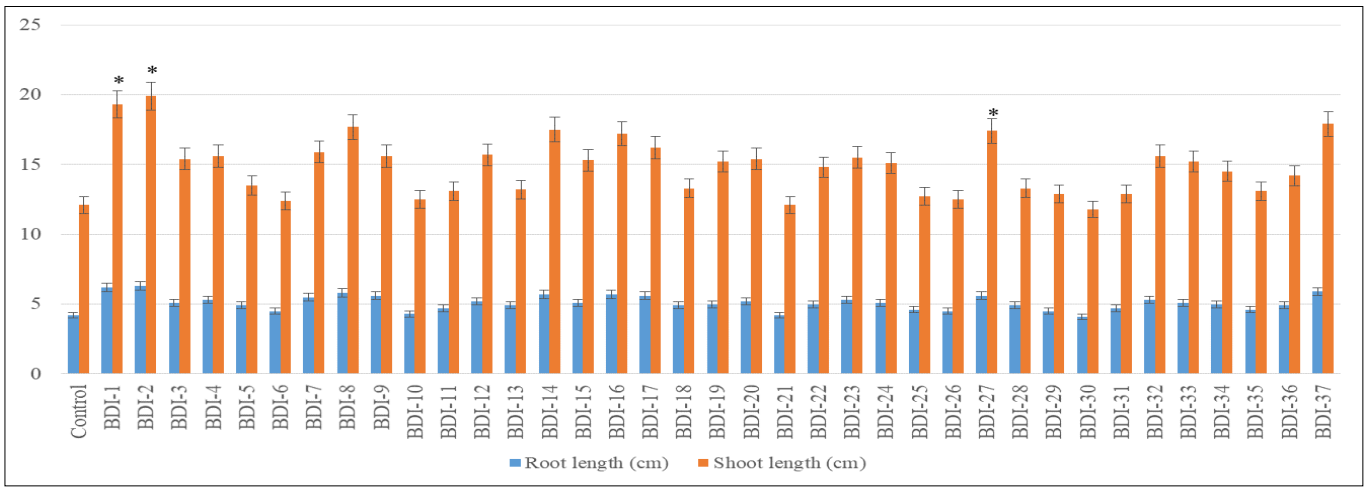

Fig. 2. The influence of wheat seeds inoculation with isolated rhizosphere bacteria on root and shoot length (10 days).

*- statistically significant at p≤0.05

Table 1. The active PGPR isolates from grapevine rhizosphere and their closest relatives from GenBank (NCBI)

Isolated bacteria deposited to GenBank			Closest match from GenBank based on 16S rRNA genes analysis		
Isolate	**Length (bp)**	**Accession number**	**Reference strains**	**Accession number**	**Percent identity**
BDI-1	1464	OP727725	*Pantoea agglomerans*	MT605811.1	99.73
BDI-2	1429	OP782582	*Priestia megaterium*	MT525296.1	99.72

Table 2. Plant growth-promoting and phenotypic characteristics of two active PGP bacterial strains

Characteristics	*Pantoea agglomerans* BDI-1	*Priestia megaterium* BDI-2
	Reaction	
Plant growth-promoting traits		
N_2-fixation	+	+
IAA (µg/mL) production	168.46	179.53
Phosphates solubilization	+	+
ACC-deaminase production	-	+
Siderophores production	+	-
Phenotypic characteristics		
Growth at 4°C	+	+
Growth at 44°C	-	+
Motility	+	+
Gelatin liquefaction	-	+
Voges-Proskauer reaction	+	-
Nitrate reduction to nitrite	+	-
Gas produced from D-glucose	-	-
Arginine dihydrolase	-	-
Phenilalanine deaminase	+	+
Catalase	+	+
Urease	-	+
Acid produced from:		
L-arabinose	+	-
D-ribose	+	+
D-xylose	+	-
D-fructose	+	+
D-mannose	+	-
L-rhamnose	+	-
L-sorbose	-	-
D-cellobiose	-	+
D-galactose	+	+
Lactose	-	+
Maltose	+	+
Melibiose	-	+
Sucrose	+	+
Trehalose	+	+
Melezitose	-	-
Raffinose	-	+
Glycerol	+	+
Meso-erithriol	-	-
Adonitol	-	-
Dulcitol	-	-
Inositol	-	+
D-mannitol	+	+
D-sorbitol	-	-
A- methyl-D-glucoside	-	-
Salicin	+	-
Inulin	-	+

Note: "+"- positive, "-"- negative

P. agglomerans BDI-1 - siderophores.

Phenotypic characteristics of bacteria

The phenotypic characteristics of bacteria *P. agglomerans* BDI-1 and *P. megaterium* BDI-2 are shown in Table 2. The strain *P. agglomerans* BDI-1 could grow at 4 °C but couldn't at 44 °C, was motile, couldn't liquefy gelatin, Voges-Proskauer reaction was positive, reduced nitrates to nitrites, couldn't produce gas from D-glucose, arginine dihydrolase phenylalanine negative, produced phenylalanine deaminase and catalase, but not urease. The strain produced acid from salicin, glycerol, trehalose, maltose, sucrose, D-mannitol, D-galactose, L-rhamnose, L-arabinose, D-mannose, D-fructose, D-xylose and D-ribose.

The strain *P. megaterium* BDI-2 grew at 4 °C and 44 °C, was motile, liquefied gelatin and produced phenylalanine deaminase, catalase and urease. The strain could not reduce nitrates to nitrites, did not produce arginine dihydrolase, the Voges-Proskauer reaction was negative and there was no gas formation from D-glucose. The strain produced acid from inulin, inositol, glycerol, sucrose, trehalose, raffinose, melibiose, maltose, lactose, D-mannitol, D-cellobiose, D-galactose, D-fructose and D-ribose.

Discussion

As a result of the isolation and screening of bacteria from the rhizosphere of a grapevine, just two out of 37 bacteria were chosen as active PGPR. The isolates BDI-1 and BDI-2 were highly efficient in wheat seed germination and PGP. It can be explained by the revealed properties of these isolates: nitrogen fixation, phosphate solubilization, production of IAA, siderophores and ACC-deaminase. Plants can get much benefit from free-living and symbiotic nitrogen-fixing bacteria because they can reduce atmospheric N_2 to NH_3 through the nitrogenase enzyme complex, making it accessible for plant uptake and fulfilling their nutritional requirements (30). Aasfar *et al.* (31) reported about improvement in wheat plant growth after seed bacterization with soil N_2-fixing bacteria. Shurigin *et al.* (32) also reported the positive effect of nitrogen -fixing bacteria on the growth of cucumber. Thus, the ability of the strains *P. agglomerans* BDI-1 and *P. megaterium* BDI-2 to fix nitrogen contributes a lot to their plant growth-promoting efficacy. Phosphorus (P) is an essential element for plant development. Phosphorus plays a key role in root development and significantly contributes to plants resistance against diseases and increasing crop yield (33, 34). Phosphorus takes part in cellular division and the structure of nucleic acids (35). Most of the phosphates remain stable in the soil due to their insoluble forms and are unavailable to plants. The phosphate solubilizing ability of bacterial strains *P. agglomerans* BDI-1 and *P. megaterium* BDI-2 is a very beneficial characteristic because they can transform insoluble inorganic phosphates to soluble forms and make phosphorus available for plants (36). The strains *P. agglomerans* BDI-1 and *P. megaterium* BDI-2 produced IAA in relatively high concentrations. IAA is one of the most active phytohormones, improving plant growth (37). IAA synthesized by rhizobacteria increases root size, weight, root hairs and lateral root number (38, 39), thus increasing the area of contact with the surrounding soil. The increased availability of soil resources in larger areas and enhanced nutrient acquisition help to improve plant growth and yield (40). Iron is necessary due to its ability to sustain the life activities of a plant. The bioavailability of iron in soil is low because it mainly presents there in the form of insoluble ferric Fe (III) ions (41). Plant growth-promoting rhizobacteria improve iron uptake, using siderophores to chelate iron, providing plants with the required amount of iron (42, 43). The production of siderophores by the strains *P. agglomerans* BDI-1 and *P. megaterium* BDI-2 is one of the important factors in improving plant growth. Different kinds of stresses (drought, salinity, etc.) are known to increase ethylene production, which can inhibit root elongation, shorten vegetation time and decrease yield (44, 45). However, it was reported that ACC-deaminase-producing PGPR can improve the growth of plants in stressful conditions by reducing the level of ethylene due to the hydrolysis of its precursor - ACC, to α-ketobutyrate and ammonia (46, 47). *P. megaterium* BDI-2 producing ACC-deaminase could reduce stresses during plant growth.

Conclusion

For the first time, this study was reported about PGPR isolated from the rhizosphere of a grapevine grown in light serozyom soils of Uzbekistan. Based on screening of 37 isolates for wheat seed germination stimulation and PGP, just two isolates, BDI-1 and BDI-2 were chosen as the most active. Based on 16S rRNA gene analysis, the strains BDI-1 and BDI-2 were identified as *Pantoea agglomerans* and *Priestia megaterium* accordingly. Both strains showed phosphate-solubilizing and nitrogen-fixing abilities and produced IAA; however, BDI-1 also produced siderophores and BDI-2-ACC-deaminase. The presence of these properties lets them show their high activity in plant growth and seed germination promotion. The bacteria *Pantoea agglomerans* BDI-1 and *Priestia megaterium* BDI-2 are promising PGPR, which can be applied as microbial inoculants for plant growth improvement.

Authors' contributions

BD and SA performed the experiments. VS wrote the draft of the manuscript. DJ statistically analyzed the results. BT and GK conducted the critical revision of the manuscript. KD worked out the concept and design, supervised and funded the experiments. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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