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Investigating Effective Factors in Improving Plant Growth by Native Phosphate-dissolving Bacteria Isolated from Wheat

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ABSTRACT

Wheat is one of the most important strategic products in providing food security for the growing world population. The purpose of this research is to isolate and identify plant growth-promoting bacteria from the wheat rhizosphere and their inhibitory effect on the growth of the pathogenic fungus *Fusarium* sp. isolated from wheat. The initial screening of 100 bacterial strains isolated from wheat roots was done based on their ability to dissolve phosphate. The effective factors in plant growth and inhibition of fungal pathogens, such as the ability to produce IAA, siderophores, protease, biofilm, bacterial swarming motility, tolerance to salt, seed germination, and seed vigor tests, were investigated. In the initial screening, four bacterial isolates with the highest amount of phosphate dissolution were identified based on morphological and biochemical characteristics as well as the sequence of 16S rRNA region belonging to *Curtobacterium* sp., *Arthrobacter* sp., *Acinetobacter calcoaceticus*, and *Cellulosimicrobium* sp.; *Curtobacterium* sp. had the highest amount of IAA (6.14µg/ml), siderophore (2.25cm), protease (1.4cm), and the highest tolerance to salinity (20%) and phosphate dissolution (375.3mg/ml). In the swarming motility test, the highest values were related to *Arthrobacter* sp. and *Curtobacterium* sp. at 2.7 and 2.2cm, respectively. The highest amount of biofilm formation was associated with *Acinetobacter calcoaceticus* and *Curtobacterium* sp. with an average absorption coefficient of 0.153 and 0.139, respectively. The highest and lowest average values of percentage inhibition of pathogenic fungus colony growth were evaluated in *Curtobacterium* sp. isolate with a value of 57.3%, and *Cellulosimicrobium* sp. isolate with 33%, respectively.

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Introduction

Wheat (*Triticum* sp.), one of the most important crops with more than 215 million hectares under cultivation and production of more than 750 million tons per year, is cultivated in a wide region of farmlands in the world, including Iran (CGIAR, 2018). This product provides more than 45% of the protein and 55% of the calories humans need. Ensuring food security of the growing populations and containing the environmental hazards posed by the use of chemical pesticides (toxins), salinity stresses, drought, pests, and diseases are among the most important issues and challenges facing human beings (Goswami *et al.*, 2016; Glick, 2012). Fungal pathogens can reduce agricultural production by up to 30% if not correctly managed (Goswami *et al.*, 2016). In the face of the above problems, soil biological capacity or population of soil-beneficial microorganisms, including bacteria and fungi, is useful and promising in maintaining the health of agricultural soils and reducing environmental

issues. Utilizing this biological capacity is biologically possible, ecologically and economically sustainable, and socially acceptable. The number of diverse biological bacterial populations in the plant rhizosphere is about 100 to 1000 times greater and more diverse in microorganisms than in non-rhizospheric soils (Goswami *et al.,* 2016; Glick, 2012). About 2 to 5% of plant growth-promoting bacteria (PGPB) are known as plant growth-promoting rhizobacteria (PGPR). These microorganisms enhance plant growth and fertility as well as plant tolerance to environmental stresses and pathogens (Goswami *et al.,* 2016). Direct mechanisms utilized by PGPRs include the supply of plant-absorbable phosphorus, the stabilization of atmospheric nitrogen, the separation and supply of rare elements such as iron-using siderophores, and the production of plant hormones such as gibberellins, cytokines, and auxins. Indirect effects of plant growth-promoting bacteria are achieved by increasing the plant's tolerance to biological and non-biological stresses by forming structures such as biofilms. In addition, many soil microorganisms are able to dissolve non-absorbable forms of metallic minerals through the secretion of organic acids (Van Hamet *et al.,* 2003; Idris *et al.,* 2004). These microorganisms have the potential to benefit the agricultural industry and improve the quality of crops (Rosenblueth *et al.,* 2006). Due to the limitations of pesticide use, environmental pollution, and problematic management of soil-borne pathogens, as well as the aim of increasing the fertility of strategic wheat crops, identifying and studying native and specific PGPRs of wheat is necessary and important. The purpose of the present study is to isolate and identify native bacteria of the wheat rhizosphere and screen the bacterial isolates through production tests of siderophore, biofilm, indole acetic acid, protease, phosphate solubility, swarming motility, antagonistic properties, and salinity tolerance.

Materials and Methods

Isolation of bacterial colonies

Wheat root samples and rhizospheric soil were collected (pH 7.4, 25°C) from different parts of Selseleh City of Lorestan province in the spring of 2021 (Fig 1). Since the collected soil was devoid of clods and was soft, there was no need for crushing or any other treatment. In order to isolate the bacteria, the serial dilution series method was used on nutrient agar (NA) and incubated at 28°C for 72 h (pH 7). After the growth of bacterial colonies on the culture medium, single colonies were selected based on color, size, and appearance (Schaad *et al.,* 2001; Aeini *et al.,* 2018).

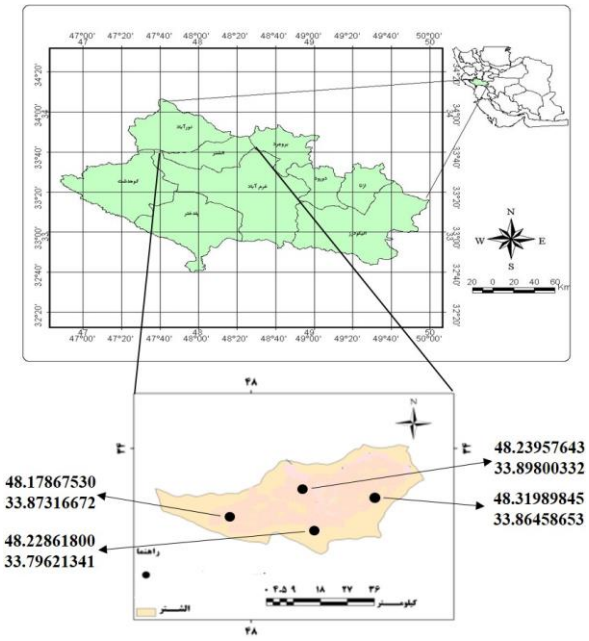

Fig. 1. Location map of the study area and sampling sites in Selseleh City. The black circle (●) shows the geographical coordinates.

Screening of bacterial isolates

Bacterial isolates were cultured on Sperber agar culture media for initial screening and then incubated for seven days at 27°C. The development of a transparent zone surrounding the bacterial colony is considered to be the positive activity of bacteria in dissolving mineral phosphate. The diameter of this clear halo was measured after one week. This test was performed in 3 replications for each bacterial isolate (Sperber, 1985). The ingredients of Sperber agar culture media include 10 g glucose, yeast extract 0.5 g, 0.1 g calcium chloride, 0.25 g magnesium sulfate, 2.5 g calcium phosphate, and 15 g agar in 1000 ml distilled water.

Moreover, to quantify phosphate solubility, bacterial species were cultured in liquid Sperber media; 50 µl of bacterial suspension with a

concentration of 10^9 CFU/ml was inoculated in liquid Sperber media. Five days after inoculation, the bacterial suspensions were centrifuged at 10,000 rpm for 10 minutes. One ml of the supernatant was mixed with 3 ml of distilled water and 1 ml of ammonium molybdate-vanadate reagent. Optical absorption intensity was read at 430 nm using a spectrophotometer after 20 minutes. The amount of soluble phosphorus in the culture medium was calculated compared to the standard curve (Sperber, 1985).

Identification of bacterial isolates

A few bacterial isolates that had the highest phosphate solubility were selected and identified based on phenotypic and biochemical tests. Various tests, including Gram staining reaction, oxidase, catalase, aerobic/anaerobic, starch hydrolysis, growth in NaCl 5, 7, 10, and 15%, HCN production, ammonia production, growth at 4, 28, 37, and 45ºC, esculin hydrolysis, nitrate reduction, gelatin hydrolysis, arginine dihydrolase, were performed based on standard bacteriological methods (Schaad, 2001). After grouping the bacterial isolates based on phenotypic and biochemical tests to identify isolates more accurately, four isolates were selected as representative, and DNA extraction was performed using Sinagen Company's DNA extraction kit. Then, the 16S rDNA gene region was amplified using general *16s-F* (5'-AGAGTTTGATCCTGGCTCAGTCG) and *16s-R* (5'-AGAGTTTGATCCTGGCTCAG) primers (Weisburg, 1991). Polymerase chain reaction was done with a final volume of 25 µl including 10 ng/µl of template DNA, 0.4 µM of each forward and reverse primer, 0.2 mM dNTP, 2 mM $MgCl_2$, 2.5 µl of 10X PCR buffer and 1 unit of Taq DNA polymerase. PCR was done under temperature conditions of 5 minutes for initial denaturing at 94˚C, 35 cycles including denaturing at 94˚C for 1 minute, annealing at 60˚C for 30 seconds, extension at 72˚C for 90 seconds, and the final extension at 72˚C for 5 minutes in a thermocycler (Eppendorf, Germany). After amplifying the expected fragment in the range of 1500 bp, the PCR product was sent to Pishgam Company for sequencing. The result was evaluated using the BLASTN tool in the Ez-Taxon database. A phylogenetic tree was drawn based on the nucleotide sequence of the 16s rDNA gene of the sequenced bacterial strains isolated from wheat rhizosphere in this study and some isolates from EZ-taxon. The tree was constructed by MEGA7 software using the Neighbor-joining method and Tamura-Nei model with 1000 replicates of the bootstrap test.

Indole acetic acid production test

Qualitative measurement to evaluate the ability of isolates to produce auxin using DF agar medium was done based on the Indole acetic acid (IAA) production method (Bric *et al.*, 1991). Quantitative measurement of auxin production capacity was performed using the Salkowski staining method (Glickmann and Dessaux, 1995). DF agar medium includes 2 g $(NH_4)_2SO_4$, 4g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4$.7H2O, 1 mg $FeSO_4.7H_2O$, 10 µg H_3BO_3, 10 µg $MnSO_4$, 70 µg $ZnSO_4$, 50 µg $CuSO_4$, 10 µg MoO_3, 2 g glucose, 2 g gluconic acid, 2 g citric acid, and 1000 ml distilled water.

Evaluation of siderophore production

Semi-quantitative detection of the ability to produce iron carrier compounds by isolates was performed using CAS-agar medium by the Alexander and Zuberer (1991) method. Isolates capable of producing iron transporters were identified based on the formation of a yellow halo. Petri dishes containing bacterial isolates were stored at 28˚C and performed in 3 replications. Briefly, CAS-agar medium mg 60.5 Ezrol chrome composition was dissolved in 50 ml of sterile water and then mixed with 10 ml of iron (III) solution (including 1mmol/L $FeCl_3.6H_2O$ in 10 mmol/L HCl). This solution was slowly mixed with 72.9 mg hexadecyl tri methyl ammonium bromide (HDTMA), which was previously dissolved in 40 ml of sterile water, and then mixed with 900ml of sterile NB medium containing 15 g/L of agar.

The ability to produce proteases

Protease activity was measured by inoculating 20 µl of 10^8 CFU/ml population of each bacterial isolate as a spot in 3 replications on SMA (skim milk agar) culture medium. The petri dishes were incubated at 28˚C for 24 hours. Protease activity was indicated by the presence of a clear

area around the colony on the surface of the SMA medium (Pratika *et al.*, 2021).

The ability of bacterial isolates to form biofilms

Microplate ELISA was used to evaluate the quality and quantity of biofilm formation (George, 2011). In this test, a 10^8 CFU/ml population of bacterial isolates was used on LB media in ELISA plate wells. Then, the plates were placed in an incubator shaker for 24 hours at 60 rpm. After 2 hours, the plates were incubated with 1% violet crystal solution for 15 minutes, and after washing, the biofilm structures were evaluated. Consequently, the plates were quantitatively examined using a 30% acetic acid solution and measuring the amount of light absorption at 550 nm with a spectrophotometer. This test was performed in 3 replications, and the results were statistically analyzed by SAS ver. 9.2 software.

Bacterial swarming motility test

NB medium was used to perform a swarming motility test. After preparation of 10^8 CFU/ml suspension from each bacterial isolate, extracellular polysaccharides were removed by washing with PBS buffer in 3 steps. Then, 20 µl of each bacterial isolate was placed on 6 mm paper disks on NA media containing 0.6% agar (Narimani *et al.*, 2017). The test was done in 3 replications, and the bacterial motility was measured for one week, and the results were statistically analyzed by SAS ver. 9.2 software.

Salt tolerance

This test was performed due to the importance of osmotic pressure tolerance in bacteria. For this purpose, agar culture media containing bacterial isolates identified up to 7 salinity levels, including 6%, 7%, 8%, 9%, 10%, 15%, and 20% sodium chloride (NaCl), were examined for in vitro bacterial growth. Bacterial growth was evaluated after 24 to 48 hours at different salt concentrations (Bano and Mussarat, 2009).

Germination ability and seed germination

NB medium was used to inoculate bacterial isolates. A suspension of 1×10^7 CFU/ml population was prepared from each isolate. After disinfecting the seeds with 70% ethanol and washing them with sterile distilled water, the seeds were inoculated into a bacterial suspension for 30 minutes. The seeds were placed in a growth chamber at 22˚C and a light period of 16 hours. The experiment was performed in a completely randomized design with three replications and four different bacteria. In order to measure the germination rate, germinated seeds were counted every 12 hours. The seed was considered germinated only when the root length reached 2 mm. Germination percentage and vigor index were measured at the end of the 10th day. The germination rate (Abdul Baki and Anderson, 1973) and vigor index (Maguire, 1962) were calculated using the following equations:

$$\text{Germination speed}=\frac{\sum(\text{Germinated seeds in each time counting})}{\text{Counting time}}$$

Vigor index= (Average root length+ Average stem length) × Germination percentage

Biological control test

Fusarium species were used to measure the antifungal activity of isolates. After re-culturing this fungus in PDA medium, a block of fungus was added to the center of a new culture medium, and 10 µl of four bacterial isolates with a concentration of 1×10^7 CFU/ml^{-1} with three replications was cultured on all four sides of the fungal disk at a distance of 2.5 cm linearly. The petri dishes were kept in an incubator at 28˚C, and the fungal colony diameter was measured after seven days and compared with the control. The percentage of inhibition of each isolate was calculated using the following formula (Moreira *et al.*, 2014):

$$\text{Percentage of inhibition}=\frac{\text{Mean fungal growth in control}-\text{Mean fungal growth in treatment}}{\text{Mean fungal growth in control}}$$

Statistical analysis

All experiments were performed in a completely randomized design with three replications per treatment. The initial data were entered into Excel software, and statistical analysis was performed using SAS software in a completely randomized design with three replications. The means were compared based on Duncan's multi-domain method at a probability level of 5%.

Results

Identification of isolates

In the initial isolation, 100 bacteria were isolated from the soil samples. Ultimately, the top 4 isolates in terms of phosphate dissolution rate were selected and identified. The results of the phenotypic-biochemical tests conducted to identify the bacteria extracted from the soil are briefly presented in Table 1. *Curtobacterium* sp. is a Gram-positive bacterium that is obligate aerobe, negative oxidase, and positive catalase. It is capable of hydrolyzing casein, esculin, and gelatin. This strain can grow in 20% salt concentration. However, it cannot hydrolyze starch, HCN production, nitrate reduction, and arginine dihydrolase. *Arthrobacter sp.* is a Gram-positive bacterium that is obligate aerobe, negative oxidase, positive catalase, and positive nitrate reduction.

Table 1. Phenotypic-biochemical characteristics of bacteria isolated from wheat rhizosphere.

Characteristics \ Isolates	*Curtobacterium* sp.	*Arthrobacter* sp.	*Acinetobacter calcoaceticus*	*Cellulosimicrobium* sp.
Gram stain	+	+	-	+
Type of metabolism	O	O	O	F
Oxidase	-	-	-	+
Catalase	+	+	+	+
Starch hydrolysis	-	-	-	+
Growth in NaCl (5 %)	+	+	-	+
(7 %)	+	+	-	+
(10 %)	+	-	-	+
(15 %)	+	-	-	+
HCN production	-	-	-	-
Casein hydrolysis	+	+	+	+
Ammonia production	+	+	+	+
Growth at 4 ◦C	-	-	-	-
28 ◦C	+	+	+	+
37 ◦C	+	+	+	+
45 ◦C	-	+	-	-
Nitrate reduction	-	+	-	+
Esculin hydrolysis	+	+	-	+
Gelatin hydrolysis	+	+	-	+
Arginine dihydrolase	-	-	-	-

O: indicated that oxidation; F: indicated that fermentation

It is capable of hydrolyzing casein, esculin, and gelatin. This strain can grow in 8% salt concentration and is not capable of hydrolyzing starch, HCN production, and arginine dihydrolase. *Acinetobacter calcoaceticus* is a Gram-negative bacterium that is obligate aerobe, negative oxidase, positive catalase, and positive nitrate reduction. However, this strain is incapable of starch hydrolyzing, HCN production, esculin hydrolase, gelatin hydrolase, and arginine dihydrolase; it has the ability to hydrolase casein and produce ammonia. *Cellulosimicrobium sp.* is a Gram-positive bacterium that is anaerobe, positive oxidase, positive catalase, negative HCN production, and negative arginine dehydrolase. This strain is capable of starch hydrolase, esculin hydrolase, gelatin hydrolase, casein hydrolase, ammonia production, and nitrate reduction.

To identify bacterial isolates, in addition to phenotypic and biochemical tests, a 1500 bp fragment was amplified using 16s-F and 16s-R primers designed based on the 16S rDNA region (Fig. 2). The isolates were identified based on the results of phenotypic and biochemical tests, as well as the results of sequencing and the 16s-based ID in the EZ-TAXON database. Based on phenotypic-biochemical characteristics and sequencing of the 16S rDNA partial gene region, isolates of *Curtobacterium* sp., *Cellulosimicrobium* sp., *Arthrobacter* sp., and *Acinetobacter calcoaceticus* were identified (Table 1). After using the 16s-based ID in the EZ-TAXON database, the bacterial isolate *Curtobacterium* sp. (An. OP975661) had the most similarity of 97.36% with isolates *Curtobacterium flaccumfaciens* (An. AJ312209). Additionally, *Cellulosimicrobium* sp. (An. OP965541) with 95.73% had the most similarity with *Cellulosimicrobium cellulans* (An. CA0101000359), *Arthrobacter* sp. (An. OP975659) with 95.19% had the most similarity

with *Arthrobacter pascens* (An. X80740), and *Acinetobacter calcoaceticus* with accession number OP846049 showed the highest similarity of 97.24% with *Acinetobacter calcoaceticus* (An. AIEC01000170) (Fig. 3).

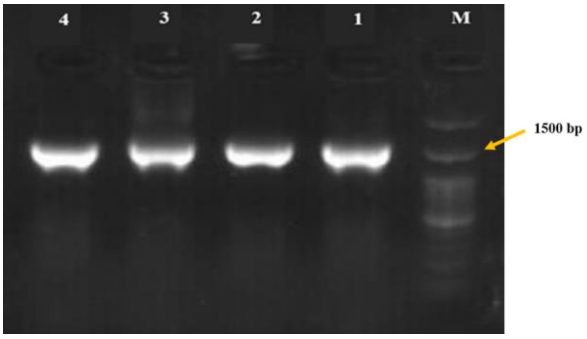

Fig. 2. Electrophoresis pattern of PCR products on 1% agarose gel. M= Molecular marker (3000 bp); 1= *Curtobacterium* sp.; 2= *Arthrobacter* sp.; 3= *Acinetobacter calcoaceticus*; 4= *Cellulosimicrobium* sp.

Indole acetic acid production test

The amount of IAA production by isolates varied from 1.92 to 6.14± 0.7 µg/ml. The highest amount of IAA production is related to *Curtobacterium* sp. with a production of 6.14± 0.7 µg/ml, and the lowest amount is associated with *Cellulosimicrobium* sp. with a production of 1.92 µg/ml. The amount of IAA produced by each isolate is shown in Table 2.

Evaluation of siderophore production

The results of the qualitative test on the CAS-agar culture medium showed that four species of *Curtobacterium* sp., *Cellulosimicrobium* sp., *Acinetobacter calcoaceticus*, and *Arthrobacter* sp. can produce siderophore. The appearance of a yellow halo around the bacterial colony indicates the production of siderophore (Fig. 4A1 and A2). In this test, the ratio of the halo diameter to the colony diameter was variable in the isolates, and the highest siderophore production ability (highest ratio to the halo diameter) after seven days belonged to the isolate of *Curtobacterium* sp. with a ratio of 2.25± 0.3 cm. Four species that had the ability to produce siderophore were compared quantitatively. In this study, *Curtobacterium* sp. showed the highest amount of light absorption at the wavelength of 630 nm with an average of 0.23.

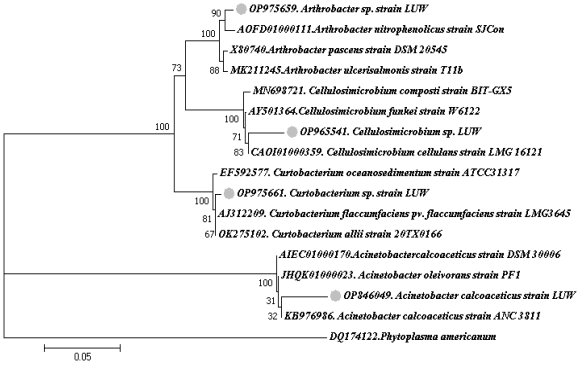

Fig. 3. Phylogeny tree of bacterial isolates isolated from wheat rhizosphere soil. ●= The isolate sequenced in this study; The bottom bar of the phylogenetic tree shows the evolutionary distances as 0.05 (5%) change in each nucleotide position.

The ability of isolates to produce protease

In the qualitative examination of the protease production test, 100 µl of each sample was cultured on CAS-agar solid culture medium and incubated for 24 hours at 37ºC. The largest transparent halo created by the *Curtobacterium* sp., *Arthrobacter* sp., *Acinetobacter calcoaceticus*, and *Cellulosimicrobium* sp. isolates had average values of 1.4± 0.22, 1.1± 0.16, 0.5± 0.05, and 0.3± 0 cm, respectively (Fig. 4B1 and B2).

The ability of bacterial isolates to form a biofilm

In order to check the amount of biofilm formation by the isolates, the microplate ELISA method was used. After staining, the biofilm structure was evaluated using an optical microscope (Fig. 4C1 and C2). The blue color in the figure shows the formation and attachment of the biofilm to the well. The biofilm structure in *Acinetobacter calcoaceticus* had the highest degree of density and expansion. Moreover, the lowest density and spread of biofilm among the species were related to *Cellulosimicrobium* sp. Consequently, in order to quantitatively evaluate the ability of isolates to form biofilm, 30% acetic acid was used at a wavelength of 550 nm.

Table 2. Variance analysis of effective factors in plant growth and inhibition of fungal pathogen.

		Average of Squares								
Sources of changes	**Degrees of freedom**	**IAA**	**Siderophore**	**Protease**	**Biofilm**	**Swarming**	**Quantitative dissolution of phosphate**	**Qualitative dissolution of phosphate**	**Colony growth**	**Growth inhibition**
Treatment	4	16.80**	0.02**	1.03**	0.01**	3.18**	0.0007**	0.51**	2.34**	1379.93**
Rep	2	0.02^{ns}	0.0001^{ns}	0.01^{ns}	0.0001^{ns}	0.002^{ns}	0.00001^{ns}	0.02^{ns}	0.04^{ns}	3.26^{ns}
C.V.	***	4.41	18.14	15.12	15.12	17.94	13.94	21.62	8.43	15.02

* and ** are significant at 5 and 1% levels, respectively, ns: non-significant level.

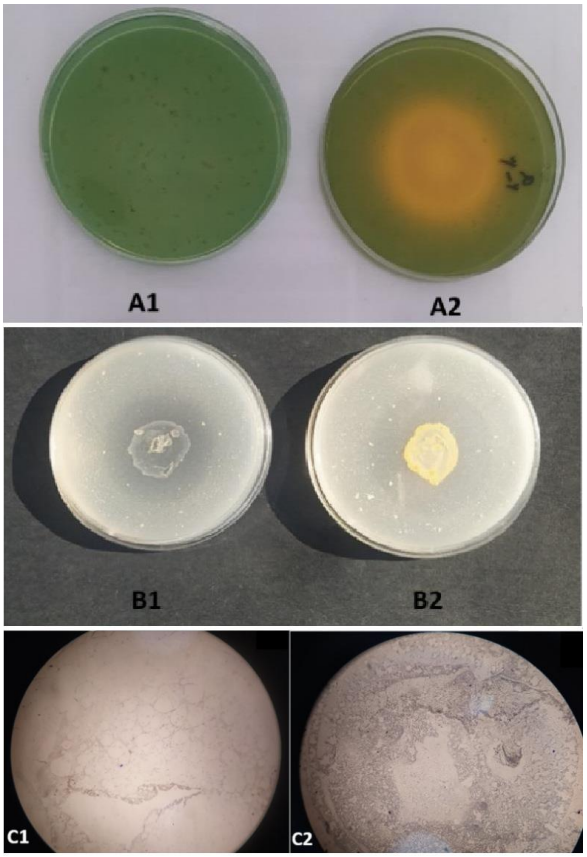

Fig. 4. Image of siderophore production, protease production, and the formation of bacterial biofilm: A1 and A2 are siderophore production (appearance of yellow halo) by control and *Curtobacterium* sp. on CAS-agar culture medium, respectively; B1 and B2 are protease production (appearance of clear halo) of *Arthrobacter* sp. and *Curtobacterium* sp., respectively; C1 and C2 are the formation of bacterial biofilm and its structure of *Cellulosimicrobium* sp. and *Acinetobacter calcoaceticus* by using the microplate ELISA method, respectively.

The comparison of the averages among the species used showed that *Acinetobacter calcoaceticus* and *Curtobacterium* sp. had the highest amount of biofilm production with an average absorption coefficient of 0.153 and 0.139, respectively, at a wavelength of 550 nm. Also, *Arthrobacter* sp. and *Cellulosimicrobium* sp. had average absorption coefficients of 0.072 and 0.055, respectively (Tables 2 and 3).

The swarming motility of bacterial isolates

Investigating the amount of swarming motility of the isolates on the 1% NA culture medium showed they were different in terms of movement speed; it also showed different forms of mobility (Fig. 5A1 and A2). The highest motility among the isolates was related to *Arthrobacter* sp. and *Curtobacterium* sp. with average values of 2.7± 0.2 and 2.2± 0.1 cm, respectively (Tables 2 and 3).

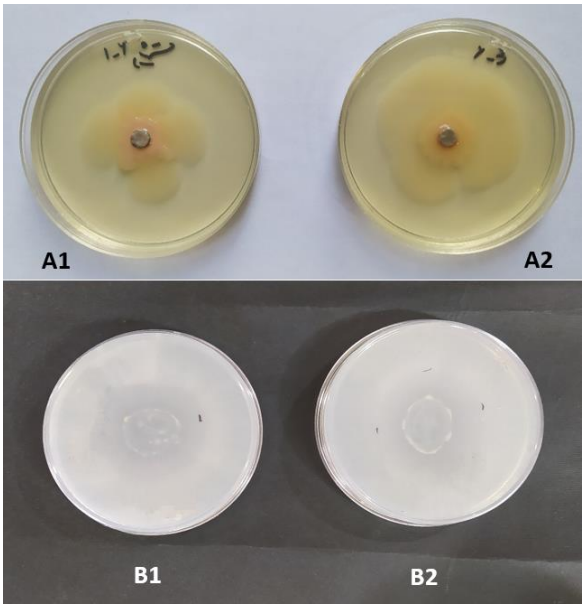

Fig. 5. Bacterial swarming motility and ability of phosphate dissolution: A1 and A2 are swarming motility of *Cellulosimicrobium* sp. and *Arthrobacter* sp. on 0.5% NA culture medium, respectively; B1 and B2 are the ability of phosphate dissolution (the clear halo around bacterial colony) of *Arthrobacter* sp. and *Curtobacterium* sp. on Sperber agar culture medium, respectively.

The dissolution ability of insoluble mineral phosphate

The results showed that all the isolates had the ability to dissolve inorganic phosphate in the

solid culture medium, and the highest and the lowest production of the phosphate dissolving halo of 1.1± 0.16 and 0.51± 0.06 cm, respectively, belonged to the *Curtobacterium* sp. and *Acinetobacter calcoaceticus* isolates. Moreover, the *Curtobacterium* sp. and *Arthrobacter* sp. isolates with 375.3± 20 and 359.6± 17 mg/ml of phosphorus, respectively, showed the highest phosphate solubility on liquid Sperber medium (Fig. 5B1 and B2) and (Tables 3 and 2).

Biological control test

Examining the ability of bacterial isolates in the cross-culture test showed that all four bacterial isolates could prevent the growth of the pathogenic fungus colony, showing a significant difference compared to the control. The average inhibition percentage of pathogenic fungus colony growth was evaluated in *Curtobacterium* sp. with the highest value of 57.3% and the lowest value belonging to *Cellulosimicrobium* sp. with 33%, respectively (Tables 2 and 3).

Salt tolerance

The results of the tolerance of the isolates to salinity showed that among the four isolates, all except *Acinetobacter calcoaceticus* were able to tolerate different levels of salinity. *Curtobacterium* sp. tolerated 20% salinity, *Cellulosimicrobium* sp. 15%, and *Arthrobacter* sp. 8% salinity.

Germination ability and seed germination

All isolates had a higher germination speed than the control. Based on Table 4, there was a significant difference between the bacteria and control treatments in terms of seed germination percentage.

There was a significant difference between isolates in terms of vigor index; this index varied from 939.45± 44 to 686.31± 0. The vigor index of the *Curtobacterium* sp. isolate (939/45) was significantly higher than the control (686/31) (Tables 4 and 5).

Table 3. Comparison of average of effective factors in plant growth and inhibition of fungal pathogen.

Sources of changes	IAA	Siderophore	Protease	Biofilm	Swarming	Quantitative dissolution of phosphate	Qualitative dissolution of phosphate	Colony growth	Growth inhibition
Arthrobacter	3.91c	0.19b	1.10b	0.07b	2.73a	0.024b	0.20c	2.26c	44.66b
Curtobacterium	6.14a	0.23a	1.43a	0.13a	2.20ab	0.014c	1.10a	1.73d	57.33a
Acinetobacter	4.36b	0.11c	0.50c	0.15a	1.73bc	0.044a	0.51b	2.46bc	39.33bc
Cellulosimicrobium	1.92d	0.07c	0.30d	0.05b	1.43c	0.025b	0.46b	2.73b	33c
Control	0e	0d	0e	0c	0d	0d	0d	4.10a	0d

The averages with common letters in the same column have a significant difference at the 5% probability level.

Table 4. Variance analysis of bacterial isolates in vigor index, germination rate, germination percentage, root and shoot length of wheat in growth chamber.

		Average of Squares								
Sources of changes	**Degrees of freedom**	**Root length**	**Stem length**	**Root wet weight**	**Stem wet weight**	**Root dry weight**	**Stem dry weight**	**Germination percentage**	**Germination rate**	**Vigor index**
Treatment	4	1.97^{**}	0.11^{ns}	0.02^{**}	0.002^{**}	0.0001^{**}	0.00007^{**}	16.76^{**}	7.86^{**}	32935^{**}
Rep	2	0.14^{ns}	0.07^{ns}	0.001^{ns}	0.0002^{ns}	0.000004^{ns}	0.000007^{ns}	0.86^{ns}	0.007^{ns}	4864^{ns}
C.V.	***	4.50	8.18	2.76	2.93	4.97	3.01	0.63	0.69	5.58

* and ** are significant at 5 and 1% levels, respectively, ns: non-significant level.

Table 5. Comparison of the average of bacterial isolates in germination index, germination rate, germination percentage, root and shoot length of wheat in growth chamber.

Sources of changes	Root length	Stem length	Root wet weight	Stem wet weight	Root dry weight	Stem dry weight	Germination percentage	Germination rate	Vigor index
Arthrobacter	4.9b	4.06a	0.50c	0.67bc	0.06ab	0.066b	98a	16.51a	896.32a
Curtobacterium	5.4a	4.26a	0.57b	0.67ab	0.06a	0.074a	96.33b	15.78b	939.45a
Acinetobacter	4.6b	3.96a	0.75a	0.71a	0.06b	0.071a	95.33bc	14.46c	826.93b
Cellulosimicrobium	3.7c	3. 73a	0.57b	0.63c	0.04d	0.064bc	94.33c	13.52d	715.78c
Control	3.5c	3.96a	0.56b	0.69ab	0.05c	0.061c	91.66d	12.54e	686.31c

The averages with common letters in the same column have a significant difference at the 5% probability level.

Discussion

Today, due to the excessive use of chemical fertilizers and environmental pollution caused by their excessive utilization for farm management, the main goal of research is to find suitable alternative methods that are efficient and compatible with the environment. Proposed alternative methods may involve issues such as the production of plant hormones, competition

for food and space, colonization of plant tissues, cell wall decomposing enzymes, secretion of antifungal compounds, induction of systemic resistance, as well as the use of PGPRs in increasing the quantitative and qualitative growth of crops and fighting disease-causing agents by making food elements such as phosphorus, iron, nitrogen, and sulfur available (Bhattacharyya and Jha, 2012). In this research, 100 native bacterial isolates were isolated from the wheat rhizosphere. Four isolates with the highest phosphate dissolution rate were selected, and their phenotypic-biochemical characteristics and 16s rDNA gene sequence were investigated. In this study, the bacterial strains of *Curtobacterium* sp., *Cellulosimicrobium* sp., *Acinetobacter calcoaceticus*, and *Arthrobacter* sp. were introduced as superior isolates. In previous studies, *Arthrobacter* sp., *Curtobacterium* sp., *Acinetobacter*, and *Cellulosimicrobium* sp. have been introduced as PGPRs (Alsharif *et al.,* 2020; Presta *et al.,* 2016; Vanissa *et al.,* 2020). Similar to the present research, Díez-Méndez and Rivas introduced the strain of *Curtobacterium herbarum* as a bacterium capable of producing siderophore, IAA, and dissolving phosphate. In their study, this strain increased the length of the saffron filaments and overall saffron production in comparison with the control plants (Díez-Méndez and Rivas, 2017). Strains of *Curtobacterium oceanosedimentum* and *Curtobacterium* sp. SAK1 were introduced with the ability to produce ammonia and IAA, along with siderophore production and phosphate solubilization by Patel *et al*. (2022) and Khan *et al*. (2019).

In previous investigations, *Acinetobacter calcoaceticus* and *Curtobacterium* sp. had the highest rate of biofilm formation. There is much evidence showing better effectiveness of PGPRs with high biofilm formation compared to other bacteria. Also, biofilm formation by PGPRs is an important strategy in adaptation for survival and colonization ability in the rhizosphere of plants (Ansari and Ahmad, 2018; Kasim *et al.,* 2016). The results of cross-culture tests on the mycelium growth of a pathogenic fungus under laboratory conditions showed that four bacterial isolates had an inhibitory effect on the growth of the pathogenic fungus, and the colony diameter decreased compared to the control. Research shows that these effects can be due to the secretion of enzymes that break down the cell wall, the production of siderophores, and volatile and non-volatile antifungal compounds (Aydi-Ben Abdallah *et al.*, 2017).

The results showed that four bacterial strains could produce the protease enzyme. These enzymes have the ability of biocontrol against pathogenic fungi and are involved in the decomposition of the cell wall of pathogenic fungi (Aydi-Ben Abdallah *et al.,* 2017). Siderophore production is also considered one of the important mechanisms of bacteria in the biological control of plant diseases. The isolates tested in this research could produce siderophore, which is vital in the microbial interactions of the rhizosphere as it absorbs iron and prevents pathogenic agents from accessing and obtaining iron (Eisendle *et al.,* 2004). Research has shown that the siderophore produced by *Pseudomonas putida* suppresses the pathogenic fungus *Fusarium oxysporum* (Kloepper *et al.,* 1980). Plant hormones of microbial origin are important compounds in increasing plant growth and tolerance to environmental stress and increasing resistance to pathogens (Yasmin *et al.,* 2017). The indole acetic acid hormone increases the proliferation and development of plant roots and thus increases host resistance to pathogens (González-Lamothe *et al.,* 2012). Biocontrol agents increase the amount of plant access to nutrients by the ability to fix nitrogen and phosphate solubility (Alori *et al.,* 2017).

The ability to produce indole acetic hormone and dissolve phosphate was observed in all four isolates. According to the production of plant growth-improving factors such as phosphate dissolution, biofilm formation, siderophore production, and IAA hormone production by the examined isolates, all four bacterial isolates caused more growth of wheat seedlings compared to the control. The results of this research are similar to the results of Díez-Méndez and Rivas (2017), Patel *et al.* (2022), and Khan *et al.* (2019).

Conclusion

In this study, bacterial strains of *Curtobacterium* sp., *Cellulosimicrobium* sp., *Arthrobacter* sp.,

and *Acinetobacter calcoaceticus* were introduced as superior isolates. Understanding the mode of action, diversity, and ecological distribution and their interaction with the environment is important for the compatibility of these factors and their success in increasing plant growth in the direction of healthy plant production. Considering the high efficiency of the present bacterial strains in the production of factors effective in plant growth, as well as the ability of their antifungal activity, they can be considered a suitable option in the production of healthy plants.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

References

Abdul-Baki, A. A., & Anderson, J. D. (1973). Vigor determination in soybean seed by multiple criteria 1. *Crop Science*, *13*(6), 630-633. http://dx.doi.org/10.2135/cropsci1973.0011183X001300060013x

Aeini, M., Khodakaramian, G., & Mirzaei Najafgholi, H. (2018). Sugar beet leaf culturable endophytic bacterial composition from the major sugar beet growing areas in the west of Iran. *Journal of Genetic Resources*, *4*(2), 105-113. https://dx.doi.org/10.22080/jgr.2019.15537.1118

Alexander, D. B., & Zuberer, D. A. (1991). Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. *Biology and Fertility of Soils*, 12, 39-45. https://doi.org/10.1007/BF00369386

Alori, E. T., Glick, B. R., & Babalola, O. O. (2017). Microbial phosphorus solubilization and its potential for use in sustainable agriculture. *Frontiers in Microbiology*, *8*, 971. https://doi.org/10.3389/fmicb.2017.00971

Alsharif, W., Saad, M. M., & Hirt, H. (2020). Desert microbes for boosting sustainable agriculture in extreme environments. *Frontiers in Microbiology*, *11*, 496411. https://doi.org/10.3389/fmicb.2020.01666.

Ansari, F. A., & Ahmad, I. (2018). Biofilm development, plant growth promoting traits and rhizosphere colonization by *Pseudomonas entomophila* FAP1: a promising PGPR. *Advances in Microbiology*, *8*(03), 235-251. https://doi.org/10.4236/aim.2018.83016

Bano, A., & Fatima, M. (2009). Salt tolerance in Zea mays (L). following inoculation with *Rhizobium* and *Pseudomonas*. *Biology and Fertility of Soils*, *45*, 405-413. http://dx.doi.org/10.1007/s00374-008-0344-9

Aydi-Ben Abdallah, R., Mejdoub-Trabelsi, B., Jabnoun-Khiareddine, H., & Daami-Remadi, M. (2017). Use of endophytic bacteria naturally associated with *Cestrum nocturnum* for Fusarium wilt biocontrol and enhancement of tomato growth. *Tunisian Journal of Plant Protection*, *12*, 15-40. https://doi.org/10.4172/1948-5948.1000259

Bhattacharyya, P. N., & Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology*, *28*, 1327-1350. https://doi.org/10.1007/s11274-011-0979-9

Bric, J. M., Bostock, R. M., & Silverstone, S. E. (1991). Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. *Applied and Environmental Microbiology*, 57(2), 535-538. https://doi.org/10.1128/aem.57.2.535-538.1991

CGIAR report (2018). *Research program on wheat*. https://hdl.handle.net/10568/103691

Díez-Méndez, A., & Rivas, R. (2017). Improvement of saffron production using *Curtobacterium herbarum* as a bioinoculant under greenhouse conditions. *AIMS Microbiology*, *3*(3), 354-364. http://dx.doi.org/10.3934/microbiol.2017.3.354

Eisendle, M., Oberegger, H., Buttinger, R., Illmer, P., & Haas, H. (2004). Biosynthesis and uptake of siderophores is controlled by the PacC-mediated ambient-pH regulatory system in *Aspergillus nidulans*. *Eukaryotic Cell*, *3*(2), 561-563. https://doi.org/10.1128/EC.3.2.561-563.2004

Glick, B. R. (2012). Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*, *2012*, 963401. https://doi.org/10.6064/2012/963401

Glickmann, E., & Dessaux, Y. (1995). A critical examination of the specificity of the

Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Applied and Environmental Microbiology*, *61*(2), 793-796. https://doi.org/10.1128/aem.61.2.793-796.1995

González-Lamothe, R., El Oirdi, M., Brisson, N., & Bouarab, K. (2012). The conjugated auxin indole-3-acetic acid-aspartic acid promotes plant disease development. *Plant Cell*, *24*(2), 762-777. https://doi.org/10.1105/tpc.111.095190

Goswami, D., Thakker, J. N., & Dhandhukia, P. C. (2016). Portraying mechanics of plant growth promoting rhizobacteria (PGPR): A review. *Cogent Food and Agriculture*, *2*(1), 1127500. https://doi.org/10.1080/23311932.2015.1127500

Idris, R., Trifonova, R., Puschenreiter, M., Wenzel, W. W., & Sessitsch, A. (2004). Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. *Applied and Environmental Microbiology*, *70*(5), 2667-2677. https://doi.org/10.1128/aem.70.5.2667-2677.2004

Kasim, W. A., Gaafar, R. M., Abou-Ali, R. M., Omar, M. N., & Hewait, H. M. (2016). Effect of biofilm forming plant growth promoting rhizobacteria on salinity tolerance in barley. *Annals of Agricultural Sciences*, *61*(2), 217-227. https://doi.org/10.1016/j.aoas.2016.07.003

Khan, M. A., Asaf, S., Khan, A. L., Ullah, I., Ali, S., Kang, S. M., & Lee, I. J. (2019). Alleviation of salt stress response in soybean plants with the endophytic bacterial isolate *Curtobacterium* sp. SAK1. *Annals of Microbiology*, *69*(8), 797-808. https://doi.org/10.1007/s13213-019-01470-x

Kloepper, J. W., Leong, J., Teintze, M., & Schroth, M. N. (1980). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature*, *286*(5776), 885-886. https://doi:10.1038/286885a0

Maguire, J. D. (1962). Speed of germination-aid in selection and evaluation for seedling emergence and vigor. *Crop Science*, *2*, 176-177. https://doi.org/10.2135/cropsci1962.0011183X000200020033x

Moreira, R. R., Nesi, C. N., & De Mio, L. L. M. (2014). *Bacillus* spp. and *Pseudomonas putida* as inhibitors of the *Colletotrichum acutatum* group and potential to control Glomerella leaf spot. *Biological Control*, *72*, 30-37. http://dx.doi.org/10.1016%2Fj.biocontrol.2014.02.001

Narimani, S., Bazgir, E., Mirzaei Najafgholi, H. (2017). Evaluation of effective factors on survival and activity of petroleum degradation bacteria in bioremediation process. *Cellular and Molecular Researches*, 30, 91-99. https://dorl.net/dor/20.1001.1.23832738.1396.30.1.8.7

Pal, K. K., & Gardener, B. M. (2006). Biological control of plant pathogens. *Plant Health Instructor*, *2*, 1117-1142. https://doi.org/10.1094/PHI-A-2006-1117-02

Patel, M., Patel, K., Al-Keridis, L. A., Alshammari, N., Badraoui, R., Elasbali, A. M., ... & Adnan, M. (2022). Cadmium-tolerant plant growth-promoting bacteria *Curtobacterium oceanosedimentum* improves growth attributes and strengthens antioxidant system in chili (*Capsicum frutescens*). *Sustainability*, *14*(7), 4335. https://doi.org/10.3390/su14074335

Pratika, M., Ananda, M., & Suwastika, I. N. (2021). Protease activity from bacterial isolates of Nepenthes maxima reinw. ex nees. *Journal of Physics: Conference Series*, *1763* (1), 012092. http://dx.doi.org/10.1088/1742-6596/1763/1/012092

Presta, L., Fondi, M., Perrin, E., Maida, I., Miceli, E., Chiellini, C., ... & Fani, R. (2016). *Arthrobacter* sp. EpRS66 and *Arthrobacter* sp. EpRS71: draft genome sequences from two bacteria isolated from *Echinacea purpurea* Rhizospheric Soil. *Frontiers in Microbiology*, *7*, 1417. https://doi.org/10.3389/fmicb.2016.01417

Rosenblueth, M., & Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interactions*, *19*(8), 827-837. https://doi.org/10.1094/MPMI-19-0827

Schaad, N. W., Jones, J. B., & Chun, W. (2001). Laboratory guide for the identification of plant pathogenic bacteria. *American Phytopathological Society*, *50*, 812-814. https://doi.org/10.1046/j.1365-3059.2001.00635.x

Siah, A., Deweer, C., Morand, E., Reignault, P., & Halama, P. (2010). Azoxystrobin resistance of French *Mycosphaerella*

graminicola strains assessed by four in vitro bioassays and by screening of G143A substitution. *Crop Protection*, *29*(7), 737-743. https://doi.org/10.1016/j.cropro.2010.02.012

Sperber, J. I. (1958). The incidence of apatite-solubilizing organisms in the rhizosphere and soil. *Australian Journal of Agricultural Research*, *9*(6), 778-781. https://doi.org/10.1071/AR9580778

Vanissa, T. T. G., Berger, B., Patz, S., Becker, M., Turečková, V., Novák, O., ... & Ruppel, S. (2020). The response of maize to inoculation with *Arthrobacter* sp. and *Bacillus* sp. in phosphorus-deficient, salinity-affected soil. *Microorganisms*, *8*(7), 1005. https://doi.org/10.3390/microorganisms8071005

Van Hamme, J. D., Singh, A., & Ward, O. P. (2003). Recent advances in petroleum microbiology. *Microbiology and Molecular Biology Reviews*, *67*(4), 503-549. https://doi.org/10.1128/MMBR.67.4.503-549.2003

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, *173*(2), 697-703. https://doi.org/10.1128/jb.173.2.697-703.1991

Yasmin, S., Hafeez, F. Y., Mirza, M. S., Rasul, M., Arshad, H. M., Zubair, M., & Iqbal, M. (2017). Biocontrol of bacterial leaf blight of rice and profiling of secondary metabolites produced by rhizospheric *Pseudomonas aeruginosa* BRp3. *Frontiers in Microbiology*, *8*, 1895. https://doi.org/10.3389/fmicb.2017.01895