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Screening and Characterization of Plant Growth-Promoting Bacteria in the Rhizo- and Endosphere of the Cucumber

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Plant growth promoting bacteria (PGPB) are beneficial bacteria that can improve the extent or quality of plant growth via wide variety of mechanisms.To look for efficient PGPB strains having multiple activities, total eight bacterial isolates were isolated from rhizosphere and endosphere of cucumber in Iran. Eight isolates of PGPB designated as *Pseudomonas protegens* strain BASU390, *Acinetobacter calcoaceticus* strain BASU624, *Brevibacillus formosus* strain BASU625, *Paenibacillus peoriae* strain BASU645, *Arthrobacter phenanthrenivorans* strain BASU589, *Pseudomonas protegens* strain BASU 630, *Pseudomonas putida* strain BASU 205 and *Stenotrophomonas pavanii* strain BASU553 were successfully isolated and characterized through phenotypical traits and 16S rDNA gene sequencing.The bacterial isolates were subjected for different plant growth promotion activities. Subsequently, to investigate the effects of the bacterial isolates on the growth of cucumber a pot culture experiment was conducted.All of the isolates demonstrated remarkable plant growth promotion activities. Furthermore, most of isolates resulted in a significant increase in plant height, root length, and dry matter production of shoot and root of cucumber seedlings. Therefore, present study suggests that PGPB isolates viz. BASU390, BASU625, BASU645, BASU589, BASU630 and BASU205 may be used as biofertilizers to enhance the growth and productivity of cucumber.

Key words: Plant growth promoting bacteria, Phosphate solubilization, HCN, IAA

Plant growth promoting bacteria (PGPB) play a key role in supporting and/or increasing plant health and growth. The majority of plant-associated bacteria derives from the soil environment. Some of them may move to the rhizosphere and subsequently the rhizoplane of their hosts before they are able to show beneficial effects. Some rhizoplane colonizing bacteria can also penetrate plant roots, and some strains may migrate to aerial plant parts, with a decreasing bacterial density in comparison to rhizosphere or root colonizing populations[1].

Based on area of colonization, PGPB can be grouped into rhizospheric (in vicinity of root), rhizoplanic (on surface of root) and endophytic bacteria. Term 'endophytic bacteria' is referred to those bacteria that do not only colonize the rhizosphere and/or the rhizoplane but can also enter plants and colonize interior of the plant parts, viz; root, shoot or seeds without causing any harmful effect on host plant and many of them have shown plant growth-promoting effects2,1. These bacteria may promote plant growth in terms of increased germination rates, biomass, leaf area, chlorophyll content, nitrogen content, protein content, hydraulic activity, roots and shoot length, yield and tolerance to abiotic stresses like draught, flood, salinity etc.

In the last decade it has been repeatedly demonstrated that the plant interior is colonized by a range of endophytes mostly deriving from the rhizosphere and many of them have been reported to improve plant growth or health.

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Following rhizosphere colonization endophytes may colonize various plant organs[3, 4].

The use of PGPB offers an attractive way to replace chemical fertilizer, pesticides, and supplements; most of the isolates result in a significant increase in plant height, root length, and dry matter production of shoot and root of plants. PGPB help in the disease control in plants. Some PGPB especially if they are inoculated on the seed before planting, are able to establish themselves on the crop roots. PGPB as a component in integrated management systems in which reduced rates of agrochemicals and cultural control practices are used as biocontrol agents. Such an integrated system could be used for transplanted vegetables to produce more vigorous transplants that would be tolerant to nematodes and other diseases for at least a few weeks after transplanting to the field[5].

In the last few years, the number of PGPB that have been identified has seen a great increase, mainly because the role of the rhizo- and endosphere as an ecosystem has gained importance in the functioning of the biosphere. Various species of bacteria such as *Aeromonas, Azoarcus, Azospirillum, Azotobacter, Arthobacter, Bacillus, Clostridium, Enterobacter, Gluconacetobacter, Klebsiella, Pseudomonas* and *Serratia* have been identified as PGPBin rhizo- and endosphere of plants[6]. Among the diverse range of PGPB identified, *Pseudomonas* and *Bacillus* spp. have a wide distribution and are the most extensively studied.

The utilization of rhisopheric and endophytic bacteria in agricultural production depends on our knowledge of the bacteria–plant interactions and our ability to maintain, manipulate and modify beneficial bacterial populations under field conditions[7]. The study of plant-associated bacteria is important not only for understanding the ecological role of such bacteria in their interaction with plants but also for the biotechnological application of these bacteria to areas such as the plant growth promotion.

The key objective of this study was to isolate and characterizerhi sopheric and endophytic plant growth promoting bacteria of the cucumber and to screen their abilities and the possession of direct and indirect plant growth promoting attributes.

MATERIALS AND METHODS

Isolation and characterization of strains

Plant and soil samples were collected from the cucumber growing fields and greenhouses of Hamedan province in Iran. The bacterial strains were isolated from rhizosphere (using dilution method) and root and shoot endophyte of the cucumber with King'sB and nutrient agar media for *pseudomonas* and other bacteria respectively. The isolated strains were biochemically and physiologically characterized. Biochemical and physiological tests were carried out according to methods described previously[8,9.10,11]. Bacterial isolates were also molecularly characterized by 16S rDNA partial gene sequencing. All selected isolations were stored in sterile water at 4°C for further investigations.

Screening of isolates for plant growth promoting activities

Indole acetic acid (IAA) production by bacteria was carried out according to Gordon and Weber (12). Briefly, the bacteria were grown in modified nutrient broth (in 1000 ml distilled water contained: 5 g NaCl, 10 g peptone and 10 g beef extract) for 48 hours on gyratory shaker (150 rpm) at room temperature. After that 100 ¼l of culture was inoculated to 10 ml minimal salt medium (in 1000 ml distilled water contained: 1.36 g KH_2PO_4, 2.13 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$; PH 7.0) amended with 5 mM L-tryptophan (13) and grown again for 48 hours on the shaker. L-tryptophan solution was prepared as stock solution containing (in 100 ml distilled water) 10 g glucose, 1 g L-tryptophan and 0.1 yeast extract. To measure of IAA produced, 1.5 ml bacterial broth culture was centrifuged at 10.000 rpm for 10 minutes. One milliliter of the supernatant was added to 2 ml ferric chloride-perchloric acid reagent ($FeCl_3$-$HCLO_4$). After 25 minutes, the mixture was read in UV-spectrophotometer at 530 nm absorbance. The amount of IAA produced per milliliter bacterial culture was estimated using standard curve.

Phosphate solubilization

Solubilization of tri-calcium phosphate by the bacterial isolates was detected in Pikovskaya's agar mediumas described by Halder*et al*[14]. All bacterial isolates were cultured on the surface of Pikovskaya's agar medium (PVK) and phosphate

solubilizing activity was estimated after 5 to 7 days of incubation at room temperature. Amphotericin B 200 mg/L added in PKA medium to avoid fungal growth15. The presence of clear halo zone around bacterial colonies after incubation time was used for positive P-solubilization activity.

Hydrogen cyanide production

Screening of bacterial isolates for hydrogen cyanide (HCN) production was performed according to Lorck method[16] modified by Alstrom and Burns[17]. Briefly, nutrient broth was amended with 4.4 g glycine/l and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 impregnated with 0.5% picric acid (yellow) and 2.0% sodium carbonate was placed in the lid of each Petri dishes. Plates were sealed with parafilm and incubated at 28 °C for 4 days. Development of yellow to orange, brown, or reddish brown was recorded as an indication of weak, moderate, or strongly cyanogenic potential, respectively.

Production of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72 h at 36 ± 2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production[18].

Enzymatic activities

Different enzymatic activities were tested; Protease activity of bacterial strains was performed according to Rodarte *et al*[19] method. The bacteria were spotted on plates of SMA medium (in 1000 ml distilled water contained: 15 g skim milk, 0.5 g yeast extract, 9.13 g agar) and incubated at 27°C for 48 h. The diameters of clear halo zone around the bacterial colonies were measured to determine the ability of protease production. Determination of lipase enzyme activity was tested according to Carrim *et al*[20] method. For determination of pectinase, cellulose, amylase and chitinase activities Miller[21] protocol were used. A positive activities were detected by apparition of transparent halo around the colonies.

Cucumber seed bacteriorization and plant growth

Seeds of cucumber (*Cucumissativus*) cultivar Hedieh were obtained from Agricultural and Natural Resources Research Centre of Hamedan, Iran. Healthy seeds were surface sterilized by soaking each in 5% sodium hypochlorite for 5 min. They were then rinsed three times in sterile distilled water to clear them of bleach before bacterial inoculation. The bacterial isolates were grown in respective media on shaking incubator (180 rpm) at 28 ± 2°C for 48h. The surface sterilized seeds of cucumber were treated with selected bacterial strains suspension using 1% carboxymethyl cellulose (CMC) for 24h and allowed 6h to dry. Cucumber seeds were sown at 3 cm depth in 2000 g sterilized soil containing plastic pot. A control treatment was also maintained without inoculated seed. Pots were kept in greenhouse for approximately 45 days. The experiment was setup in 3 replication with 8 treatments, one positive and negative control. All seeds were germinated. After 45 days, cucumber plants were harvested. Length and wet weight of shoot and root of the cucumber pants were recorded in centimeter and gram of each plant respectively. Then plants were dried in an oven at 65°C for 3 days. After this shoots and roots dry weight were recorded in gram.

Statistical analysis

of the data was carried out using the SAS software version 9.1 with a Randomized Block Design. Comparisons of means were performed by Duncan's multiple range test (LSR) at P =0.05.

RESULTS

Isolation and characterization of PGPB

In total, eight bacterial isolates were successfully isolated from the rhizosphere and shoot and root endosphere of the cucumber plants in field and greenhouse in different areas of Hamedan province of Iran (Table 1). They were designated as BASU645, BASU205, BASU390, BASU630, BASU589, BASU625, BASU624 and BASU553. The biochemical and physiological characteristics of the isolates widely varied. All the bacterial isolates were checked for catalase, oxidase and indole production, ability to grow at 40°C in nutrient broth, hydrolysis of gelatin, urease activity, spore forming and utilization of some carbon sources according to the protocols mentioned in methods and material (Table 2). They were molecularly characterized by 16S rDNA partial gene sequencing and their accession number were allotted as KP412237 (*Pseudomonas protegens*

strain BASU390), KP412238 (*Acineto bacter calcoaceticus* strain BASU624), KP412239 (Brevi bacillus formosus strain BASU625), KP412240 (Paenibacillus peoriae strain BASU645), KP412241 (Arthrobacter phenanthrenivorans strain BASU589), KP676935 (Pseudomonas protegens strain BASU630), KP676933 (Pseudomonas putida strain BASU205) and KP676934 (Stenotrophomonas pavanii strain BASU553) by National Centre for Biotechnology Information (NCBI) GenBank.

Plant growth promoting activities

The bacterial strains isolated from cucumber rhizosphere and endo sphere are capable of acting as plant growth promoting bacteria as the results of this study shown various plant growth promoting activities of the isolated bacterial strains (table 3).

Production of Indole acetic acid: All the eight isolates were checked for Indole Acetic Acid (IAA) production test. The ability of bacteria to produce IAA in the rhizosphere and endo sphere depends on the availability of precursors and uptake of microbial IAA by plant. Production of IAA was indicated by appearance of pink colour. After qualitative analysis these isolates were subjected for quantitative production of IAA. Results showed that the range of IAA production was 115.32 to 191.87 ¼g/ml. Among all the bacterial isolates, BASU390 produced high IAA concentration (191.87 ¼g/ml), followed by BASU205 (179.53 ¼g/ml) and then BASU630

Table 1. Description of the isolated bacterial strains

Source	Isolate codes	Geographical origin in Iran	Plant part
field	BASU645	Hamedan, Nahavand	Rhizosphere
greenhouse	BASU205	Hamedan, Nahavand	Endophyte
field	BASU390	Hamedan, Kabudarahang	Rhizosphere
field	BASU630	Hamedan, Lalejin	Rhizosphere
field	BASU589	Hamedan, Kabudarahang	Endophyte
field	BASU625	Hamedan, Malayer	Rhizosphere
greenhouse	BASU624	Hamedan, Amzajer	Rhizosphere
field	BASU553	Hamedan, Bahar	Endophyte

Table 2. Physiological and biochemical characterizations of the bacterial isolates

Character	BASU 645	BASU 205	BASU 390	BASU 630	BASU 589	BASU 625	BASU 624	BASU 553
Gram reaction	+	-	-	-	+	+	-	-
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	-	+	-	-
Grow at 40 °C	+	-	-	-	-	+	-	-
Indole production	-	-	-	-	+	-	+	-
Methyl Red reaction	-	-	-	-	+	-	-	-
Vogesproskauer	+	-	-	-	+	-	-	-
Spore formed	+	-	-	-	-	+	-	-
Urease	-	+	+	+	-	-	+	-
Fluorescent on KB	-	+	+	+	-	-	-	-
Growth aerobically	+	+	+	+	+	+	+	+
Nitrate reduction	+	-	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	-	-	-	-
Gelatin hydrolysis	+	-	+	+	-	+	+	+
Utilization of:								
Citrae	-	+	+	+	+	-	+	+
Malonate	+	+	+	+	+	+	+	-
Glucose	+	-	-	-	-	-	+	-

(153.26 ¼g/ml). Whereas, BASU589 most efficient P-solubilizer was found to produced 145.65 ¼g/ml of IAA. These concentrations could be the major plants growth promoting factor.

Phosphate solubilization

Piskovskaya's agar medium were used for the detection of phosphate solubilizing microorganisms. All the bacterial isolates were screened for phosphate solublization on modified PVK agar, of which the isolates demonstrated diverse levels of phosphate solubilization activity. Six isolates showed the development of sharp phosphate solublization zones, ranging from 10 mm to 20 mm. Isolates BASU205, BASU390 and BASU589 showed highest phosphate solublizationi. e 20.0 mm, 18.0 and 14.5 mm, respectively. Three isolates i.e. BASU630 (13.0 mm), BASU645(12.0 mm) and BASU625 (10.0 mm) produced zone greater than 10 mm. Isolates BASU624 and BASU553 were negative for phosphate solublization.

Hydrogen cyanide production

Hydrogen cyanide (HCN) is a dreaded chemical produced by some plant growth promoting bacteria as it has toxic properties. All the isolates were subjected to HCN production test. Color development of yellow to reddish brown shows positive result. All of the isolates were positive with the exception of isolate BASU624. Isolates BASU630, BASU390, BASU205 and BASU645 demonstrated strong results while other isolates i.e. BASU624, BASU589 and BASU553

Table 3. Plant growth promoting attributes and different enzymatic activities of the bacterial isolates

Character	BASU 645	BASU 205	BASU 390	BASU 630	BASU 589	BASU 625	BASU 624	BASU 553
HCN production	+++	+++	+++	+++	++	+	-	++
IAA production(¼g/ml)	139.84	179.53	191.87	153.26	145.65	152.34	131.93	115.32
P-Solublization activity	++	+++	+++	++	+++	++	-	-
Motility	+	+	+	+	-	+	-	-
Resistance to streptomycine	-	-	+	+	-	-	+	+
Ammonia production	+	+	+	+	+	+	+	+
Siderophore production	+	+	+	+	+	+	+	+
Cellulase	-	-	+	-	-	-	-	-
Lipase	+	-	-	+	-	+	+	+
Protease	+	+	+	+	-	-	-	+
Chitinase	+	-	-	-	+	-	+	-
Amylase	+	+	-	+	+	-	-	-
Urease	-	+	+	+	-	+	+	-
Pectinase	+	-	-	-	-	-	-	-
Gelatinase	+	+	+	+	-	+	-	+

- Negative; + positive; ++ medium producer; +++ good producer

Table 4. Plant growth promoting effects of the isolated bacteria on cucumber plant

Treatment	shootlength plant^{-1} (cm)	Root length plant^{-1} (cm)	shootdry weight plant^{-1} (gm)	Root dry weight plant^{-1} (gm)
Control	22.3 ± 0.6^{c*}	20.6± 0.6^{c}	10.8± 0.7^{e}	1.0 ± 0.1^{d}
BASU645	35.3 ± 1.5^{a}	28.7 ± 2.1^{a}	14.5± 0.3^{d}	2.9 ± 0.1^{a}
BASU205	36.0± 2.0^{a}	28.3± 2.5^{a}	17.8± 0.3^{b}	3.0 ± 0.2^{a}
BASU390	35.7 ± 1.5^{a}	29.7 ± 1.5^{a}	19.4 ± 0.2^{a}	2.9 ± 0.1^{a}
BASU630	35.3 ± 1.2^{a}	27.1± 1.7^{ab}	17.0± 0.6^{ab}	2.5 ± 0.2^{ab}
BASU589	36.6 ± 1.1^{a}	31.3± 0.6^{a}	16.0 ± 0.6^{cb}	2.4 ± 0.4^{b}
BASU625	37.0± 1.0^{a}	30.0± 1.0^{a}	16.1± 0.1^{c}	2.8 ± 0.1^{a}
BASU624	31.3± 1.5^{ab}	27.0± 1.0^{b}	13.9± 0.4^{cd}	1.3 ± 0.1^{c}
BASU553	31.2± 2.3^{b}	21.3± 1.5^{cb}	13.8± 0.7^{d}	1.5 ± 0.3^{bc}

showed moderate results.

Production of ammonia

Ammonia production is another important trait of plant growth promoting bacteria that indirectly influence the plant growth. All of the bacterial isolates in this study were showed positive for ammonia production.

Enzymatic activities

All the results of different enzymatic activities of the isolates are summarized in Table 3. It should be noted that each strain among the eight tested have at least three positive activities, but it appears clearly that the strain BASU645 is very rich with enzymatic equipment.

Length and dry weight of shoot and root

All the bacterial isolates were checked for plant growth promoting effects on length and dry weight of shoot and root of the cucumber plants in greenhouse conditions (Table 4). The PGPB isolates significantly affected the length of cucumber shoots. Results revealed that the shoot length increased in PGPB treated plants over un-inoculated control. The highest shoot length (37.0 cm plant^{-1}) was recorded in treatment of BASU625 isolate followed by statistically at par values due to isolates BASU630 (13.0 cm plant^{-1}), BASU205 (36.0 cm plant^{-1}), BASU390 (35.7 cm plant^{-1}), BASU645(35.3 cm plant^{-1}) and BASU630(35.3 cm plant^{-1}). Isolates BASU624and BASU589 showed significantly higher shoot length over control. The bacterial isolates significantly increased the root length of cucumber seedlings. Root length ranged from 20.6 to 31.3 cm. The isolate BASU589 produced the highest root length (31.3 cm), which was statistically similar to isolate BASU630 (13.0 cm). A significant increase in shoot dry matter of cucumber plant was observed in response to PGPB isolates. The highest shoot dry matter was recorded in isolate BASU390(19.4 gm plant–1) followed by BASU205(17.8 gm plant^{-1}) and BASU630(17.0 gm plant^{-1}). The rest of the isolates showed statistically significant increase of shoot dry weight over control. A significant variation in root dry weight was observed in response to different PGPB isolates. The isolate BASU205 produced the highest root dry weight (3.0 gm plant–1) followed by BASU645, BASU390 and BASU625. In this study, all the bacterial isolates significantly increased shoot/root length and dry matter production of shoot/root of cucumber plants in greenhouse conditions.

DISCUSSION

Plant growth promoting bacteria facilitate the plant growth directly by either assisting in resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents[22].

The exact mechanism of stimulating plant growth by PGPB is not clearly detected, although several hypotheses such as suppression of deleterious organisms, solubilization of phosphate, production of phytohormones e.g. IAA and promotion of the mineral nutrient uptake are usually believed to be involved[23,24].

In the current study, beneficial bacteria were isolated from rhizosphere and endophyte of the cucumber. Isolated bacteria were screened for different plant growth promoting activities and characterized by physiological and biochemical tests. All of the bacterial isolates were positive for IAA production. Among them, two isolates BASU390 and BASU205 were found to be good producers of IAA. It has been reported in previous studies that IAA production by plant growth promoting bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability25. Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil[26].

Of eight isolates, six isolates were able to solubilize phosphate and produced sharp phosphate solublization zones in PVK agar medium. Isolates BASU390 and BASU205 were unable to solubilize phosphate, whilst these two isolates were found to be medium producer of IAA. It is important to note that higher concentrations of phosphate-solubilizing bacteria are commonly found in the rhizosphere soil as compared to non-rhizospheric soil. The production of ammoniais another important feature of PGPB for stimulating of plant growth. All the bacterial isolates in the present study were able to produce ammonia. Production of HCN was detected in all of the isolates with the exception of isolate BASU624. It is important to specify that HCN, produced by many

soil microorganisms and it is postulated to play a role in biological control of pathogens[27].

Our results suggested that PGPR are able to improve growth of cucumber plants. Isolates BASU645, BASU205, BASU390, BASU630, BASU589 and BASU625 were observed most efficient inoculations for enhancement of shoot and root length and dry matter followed by isolates BASU624 and BASU553 over control. Other workers have been reported the enhancement of shoot and root length and dry matter by inoculation of plant growth promoting rhizobacteria for cucumber[28,29,30,31]. Generally, results obtained from this study suggest that PGPB isolated from rhizosphere and endophyte of the cucumber are able to induce the production of IAA, solubilization of phosphorus and improve growth of plants. The use of PGPB as inoculants biofertilizers is an efficient approach to replace chemical fertilizers and pesticides for sustainable cucumber cultivation in Iran. Further investigations, including efficiency test under field conditions are needed to clarify the role of PGPB as biofertilizers that exert beneficial effects on plant growth and development.

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