



RESEARCH ARTICLE

Plant growth-promoting endophytic bacteria associated with *Halocnemum strobilaceum* (Pall.) M. Bieb and their plant beneficial traits

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Abstract

Halocnemum strobilaceum (Pall.) M. Bieb. is a halophytic desert plant. The plant is known for its antioxidant, antimicrobial, insecticidal and phytoremediation properties. *Halocnemum strobilaceum* grows in severe salinity and drought conditions and its' survival can be associated with activity of endophytic bacteria. The aim of the research was to reveal and study plant growth-promoting endophytic bacteria isolated from *Halocnemum strobilaceum* (Pall.) M. Bieb. The plants were collected from Kyzylkum desert in Uzbekistan. The endophytic bacteria were isolated from *Halocnemum strobilaceum* (Pall.) M. Bieb. tissues and screened for cotton (*Gossypium hirsutum* L.) growth-promoting activity. As a result the most active isolates HAST-2, HAST-7, HAST-9, HAST-10 and HAST-17 were selected. The cotton seeds' inoculation with these bacterial isolates resulted in significant improvement of seeds germination, root and shoot length, and fresh plant weight due to their ability to fix nitrogen, produce indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, siderophores and solubilize phosphates. The chosen isolates were identified using 16S rRNA gene analysis and registered in GenBank (NCBI) as *Bacillus megaterium* HAST-2, *Bacillus aryabhattai* HAST-7, *Pseudomonas plecoglossicida* HAST-9, *Pseudomonas putida* HAST-10 and *Pseudomonas chlororaphis* HAST-17. These strains can be used as bio-inoculants to improve the growth of cotton and other crops.

Keywords

Halocnemum strobilaceum, bacterial endophytes, cotton, plant growth promotion

Introduction

Halocnemum strobilaceum (Pall.) M. Bieb. is a halophytic plant (*Chenopodiaceae* family) distributed in deserts from northern Africa to western Asia (1). The plant is known for its antimicrobial activities. Its shoot fractions containing long-chain fatty acids, showed antibacterial activity against human pathogenic bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Salmonella typhimurium* (2). The crude extract of *Halocnemum strobilaceum* showed insecticidal activity against red flour beetle *Tribolium castaneum* due to high content of saponins, gallic tannins, flavonoids, antocyanins and alkaloids (3). The natural antioxidants from *Halocnemum strobilaceum*: flavonoid glycosides with quercetin, isorhamnetin, and icaritin moieties showed high activity against PC3, MCF-7 and A549 cancer cell lines (4). *Halocnemum strobilaceum* might

be promising phytoremediation species because of its' high adaptive ability to live in contaminated soil and capacity to stabilize and accumulate metals in their tissues (5).

The plant grows in severe salinity and drought conditions and its survival and growth can be associated with activity of endophytic bacteria. There are many reports about positive effect of endophytes on their host plant, such as *Nypa fruticans* (6), *Armoracia rusticana* (7), *Calendula officinalis* (8) and *Cicer arietinum* (9). They provide various beneficial effects including plant growth promotion and resistance against pathogens by producing different substances such as volatile and antifungal compounds: indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, 2,4-diacetylphloroglucinol (DAPG), LCI bacteriocin, fungal cell wall degrading enzymes (lipase, protease, chitinase, glucanase), HCN (7, 8, 10-12). Reports are on the antifungal properties of rhizospheric and endophytic bacteria isolated from *Halocnemum strobilaceum* and proved that halophytes are an important source of bacteria producing antifungal metabolites (13).

At a moment there is no available information about plant growth-promoting (PGP) properties like nitrogen fixation, production of IAA, ACC-deaminase, siderophores, phosphates solubilization of endophytic bacteria isolated from *Halocnemum strobilaceum*.

The aim of the research was to reveal and study plant growth-promoting endophytic bacteria isolated from *Halocnemum strobilaceum* (Pall.) M. Bieb.

Materials and Methods

Plant collection

In total six plants of *Halocnemum strobilaceum* (Pall.) M. Bieb. plants were collected from saline soil of Kyzylkum desert (latitude 42.3424° or 42° 20' 32.7" N, longitude 63.8527° or 63° 51' 9.7" E) in Uzbekistan during spring. The distance between growing plants were not less than 10 m to increase the potential diversity of the isolated endophytic bacteria with plant growth-promoting properties. The plants were cleaned from soil by rinsing them in sterile water.

The endophytic bacteria isolation

The collected plants (shoots and roots) were cut into 5-6 cm pieces and sterilized by keeping them in 99.9% ethanol for 2 min and 10% sodium hypochlorite for 1 min. After that they were rinsed in glasses with sterile water for 2 min (14). The pieces of plants were longitudinally cut into thin slices. The plants slices in amount of 5 g were put into tubes with 9 ml of sterile water for serial dilutions (10^1 – 10^5) and shook for 5 min. The suspensions from each dilution in the amount of 100 μ l were spread on Petri plates with Tryptic Soy Agar (TSA) and left in incubator at 30°C for 96 hr. In four days the bacterial colonies of different colour and shape were transferred and streaked on plates with TSA for purification. The outer surface of sterilized plants pieces was checked for sterility by putting them onto TSA

media and incubating during four days at 30 °C. The pure cultures of endophytic bacteria were used in screening for plant growth-promoting activity.

The screening of endophytic bacteria for plant growth promotion in Petri plates

The isolated bacterial endophytes were separately cultivated in nutrient broth medium for 96 hr. at 30 °C and cells concentration was adjusted up to 10^8 CFU/ml. The seeds of cotton plant (*Gossypium hirsutum* L.) were surface-sterilized by soaking in sodium hypochlorite (2.5%) for 3 min and rinsed in sterile water (15). Sterile seeds were inoculated with bacteria (100 seeds for each isolate) by soaking in bacterial suspension and transferred into sterile petri plates with wet filter paper for germination. The sterile nutrient broth medium was used as a control. The plates were left in dark and the day/night temperature was 25/16 °C. The rate of seeds germination was checked during 4 days and the roots length were checked in the 7th day. The best root growth and seeds germination promoting bacterial isolates were tested for cotton growth promotion in pots.

Test for plant growth promotion by bacterial endophytes in pots

The selected bacterial endophytes were cultivated in nutrient broth medium for 96 hr at 30°C and cells concentration was adjusted up to 10^8 CFU/ml. The seeds of cotton plant (*Gossypium hirsutum* L.) were surface-sterilized as per standard procedure (15). Sterile seeds were inoculated with bacteria by soaking into bacterial suspension (test) and sterile nutrient broth (control) and sown into 250 ml plastic pots with sterile light grey soil. The suspensions containing single strains as well as mixture of tested strains in equal proportions were used for inoculation. All pots were set up randomly in five replications for each bacterial strain and their mixture. Three seeds were sown into each pot. Plants were grown at 28–30 °C during the day and 18–20 °C at night and irrigated with tap water as required. In 10 days, the shoots and roots length and fresh plant weight were measured.

Tests of bacteria for plant beneficial traits

The production of indole-3-acetic acid (IAA) was tested according to the standard method (16). Bacterial suspension was adjusted to 1×10^8 CFU/ml and added into flasks with 10% TSA (17) supplemented with 5 mmol/l⁻¹ of L-tryptophan and cultivated at 30°C for 24 hr. in the dark. The grown bacteria were centrifuged at 8000 \times g for 15 min and supernatant was poured into fresh tubes. The Salkowski reagent (mixture of Fe Cl₃ - 0.5 mol/l and H₂SO₄ - 7.9 mol/l) was added in a 1:1 ratio (v/v) to supernatant and left at room temperature for 30 min in the dark. The appearance of pink color indicated the production of indole-3-acetic acid. The-IAA was measured using spectrophotometer at 530 nm. Different concentrations of IAA solutions was used to construct standard curve.

The ability of endophytes to solubilize inorganic phosphate was tested according to the standard procedure (18). The bacteria were cultured on solid NBRIP

medium (%): glucose - 1, $\text{Ca}_3(\text{PO}_4)_2$ - 0.5, MgCl_2 - 0.5, $(\text{NH}_4)_2\text{SO}_4$ - 0.01, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.025, KCl - 0.02, agar - 1.5). Plates with bacteria were incubated at 28 °C for 96 days. The formation of clear zone around colonies indicated on ability to use inorganic phosphate in the form of $\text{Ca}_3(\text{PO}_4)_2$ as a sole phosphate source.

To test the strains for nitrogen fixation assay the colonies of each endophyte were streaked onto solid nitrogen-deficient malate medium (g/l): CaCl_2 - 0.02, NaCl - 0.1, FeCl_3 - 0.01, KH_2PO_4 - 0.4, K_2HPO_4 - 0.5, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.2, $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ - 0.002, sodium malate - 5, agar - 15, pH 7.2–7.4) supplemented with 50 mg/l yeast extract. The plates were incubated at 30 °C during 96 hr and the appearance of growth indicated the ability to fix N_2 . The new grown single colonies were streaked onto plates with same medium to confirm the ability of nitrogen fixation (19).

Siderophore production was determined by using chrome azurol S (CAS) agar. Isolates were streaked onto CAS agar, incubated at 30 °C for 96 days. The appearance of orange halo around the bacterial colony indicated on production of siderophores (20).

The 1-aminocyclopropane-1-carboxylate (ACC) deaminase production by bacteria was tested based on utilization of ACC as a sole N-source. The endophytes were cultivated on basal medium supplemented with 3.0 mM of ACC. The $(\text{NH}_4)_2\text{SO}_4$ was used as a positive control and the negative control was without N source (21).

Biochemical tests of bacteria

Biochemical characteristics were determined with commercially produced kit systems (API 20 NE and API ZYM; BioMerieux). To investigate further biochemical characteristics, we used methods described (22, 23) for the following tests: the oxidase reaction, levan formation from sucrose, the egg-yolk reaction, the production of extracellular lipase, hydrolysis of gelatin and starch and denitrification. Arginine dihydrolase was determined in Muller's decarboxylase base (Difco Laboratories) (24). Nitrate reductase was revealed as described (25). Growth at different temperatures was observed in MPA medium after incubation at +4 (for 10 d) and +41 °C (for 5 d).

Carbon source utilization tests were performed in the basal medium (22, 26) by using 96-well microplates as described (27). Growth was determined at 25°C for 7 d and growth greater than the control without carbon source was considered as positive.

Bacteria identification

The bacterial DNA was isolated using standard method (28). The bacterial colonies were transferred into eppendorf tubes with 1 ml of milli-Q water. The colonies were mixed with water by shaking during 1 min and incubated at 90 °C for 20 min in a Dry Block Heater. The tubes were centrifuged at 12.000 rpm for 5 min. The isolated DNA was visualized using gel electrophoresis.

The 16S rRNA gene of the extracted DNA was analyzed using PCR with the following primers: 27F 5'-GAGTTTGATCCTGGCTCAG-3' (Sigma-Aldrich, St. Louis, Missouri, USA) and 1492R 5'-GAAAGGAGGTGATCCAGCC-3' (Sigma

Aldrich, St. Louis, Missouri, USA) (29). The PCR program was as follows: a primary heating step for 30 s at 94 °C, followed by 30 cycles of denaturation for 15 s at 94 °C, annealing for 30 s at 55 °C, and extension for 1.5 min at 68 °C, then followed by the final step for 20 min at 68 °C. The PCR products were checked by electrophoresis using GelRed.

The ABI PRISM BigDye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) was used for the sequencing. The obtained sequences were compared with the sequences of the closest relatives from GenBank of the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>).

Accession numbers

The 16S rRNA gene sequences of five chosen endophytic bacteria from *Halocnemum strobilaceum* (Pall.) M. Bieb. were deposited into GenBank under the accession numbers: OK594050 - OK594054.

Statistical analysis

The statistical significance of data was tested by the analysis of variance of Microsoft Excel 2010 package. Mean comparisons were conducted using the least significant difference (LSD) test ($P=0.05$). The average values of plant growth parameters, IAA production and the standard deviation were counted based on several replications.

Results and Discussion

A total of 20 isolates of endophytic bacteria were isolated from tissues of *Halocnemum strobilaceum* (Pall.) M. Bieb (Fig. 1). The isolates were called HAST1-HAST20 as abbreviations of first two letters from words *Halocnemum strobilaceum*.



Fig. 1. *Halocnemum strobilaceum* (Pall.) M. Bieb. in Kyzylkum desert, Uzbekistan.

Screening of endophytic bacteria for seeds germination and roots growth promotion

The bacterial isolates were checked for ability to stimulate germination of a cotton plant seeds and root growth (Table 1).

The cotton seeds' inoculation with bacterial endophytes increased the rate of germination. However, the isolates HAST-2, HAST-7, HAST-9, HAST-10 and HAST-17 appeared to be the best in stimulation of seeds germination. The seeds' inoculation with these isolates on 1st day

Table 1. The influence of cotton seeds' inoculation with isolated endophytic bacteria on seeds germination and root growth

Bacterial isolates	Rate of seeds germination (%)				Root length after 7 days of seeds' inoculation
	1 day	2 days	3 days	4 days	
Control (sterile medium)	18	43	75	100	2.5±0.2
HAST-1	20	57	81	100	2.8±0.2
HAST-2	49	95	100	100	4.6±0.3
HAST-3	18	43	78	100	2.5±0.2
HAST-4	32	65	86	100	3.1±0.3
HAST-5	25	52	84	100	2.8±0.2
HAST-6	26	64	88	100	2.8±0.2
HAST-7	58	99	100	100	5.2±0.3*
HAST-8	18	45	76	100	2.5±0.2
HAST-9	45	94	100	100	4.5±0.3
HAST-10	42	96	100	100	4.8±0.3
HAST-11	36	68	91	100	3.8±0.3
HAST-12	27	63	84	100	2.8±0.2
HAST-13	21	57	79	100	2.7±0.2
HAST-14	30	61	88	100	3.5±0.2
HAST-15	19	47	81	100	2.8±0.2
HAST-16	25	58	82	100	2.9±0.2
HAST-17	53	99	100	100	4.9±0.3
HAST-18	29	64	86	100	3.3±0.3
HAST-19	18	43	76	100	2.5±0.2
HAST-20	19	43	77	100	2.6±0.2

*- statistically significant at $P \leq 0.05$

resulted in germination of more than 42% of seeds, on the 2nd day – more than 94% and on the 3rd day – 100%. In all other cases including control, 100% of seeds germinated just on the 4th day. The same isolates showed the best results in root growth stimulation. The seeds' inoculation with isolate HAST-2 led to root elongation in 1.84, HAST-7 – in 2.08, HAST-9 – in 1.8, HAST-10 – in 1.92 and HAST-17 – in 1.96 times as compared to control. As a result of screening the isolates HAST-2, HAST-7, HAST-9, HAST-10 and HAST-17 were chosen for further study of their cotton growth-promoting activity in pot experiment.

Cotton growth promotion by bacterial endophytes in pots

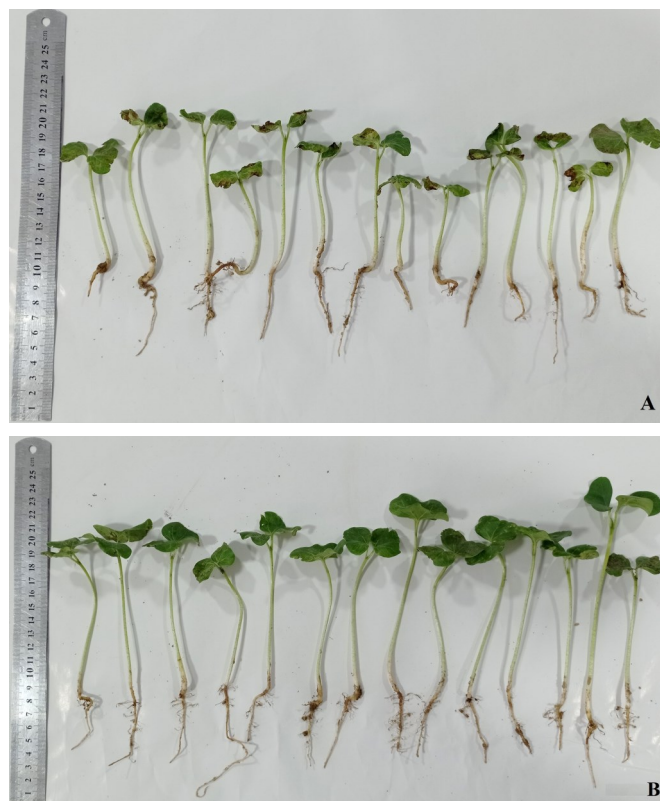
The isolates HAST-2, HAST-7, HAST-9, HAST-10 and HAST-17 were used for cotton seeds' inoculation separately (single inoculation) and in mixture (coinoculation) to check their efficiency in root and shoot growth stimulation and increase of plant fresh weight while growing in soil (Table 2).

Table 2. The influence of cotton seeds' inoculation with endophytes on plants growth in pot experiment (10 days)

Bacterial isolates	Root length	Shoot length	Plant fresh
Control (sterile medium)	3.31±0.3	8.65±0.5	1.72±0.2
HAST-2	4.49±0.4	10.98±0.6	2.12±0.2
HAST-7	5.11±0.5	12.62±0.7	2.47±0.2
HAST-9	4.18±0.3	10.43±0.6	2.03±0.2
HAST-10	4.75±0.5	11.12±0.6	2.28±0.2
HAST-17	5.05±0.5	12.71±0.7	2.55±0.3
HAST-2 + HAST-7 + HAST-9 + HAST-10 + HAST-17	5.57±0.5	14.74±0.8*	2.81±0.3

*- statistically significant at $P \leq 0.05$

The single inoculation of seeds with tested isolates increased root and shoot length and plant fresh weight. The best plant growth promoters were isolates HAST-7 and HAST-17 since they increased root length in 1.54 and 1.53, shoot length – in 1.46 and 1.47, plant fresh weight – in 1.44 and 1.48 times respectively as compared to control. The seeds' coinoculation with mixture of 5 isolates resulted in even more increase of root and shoots length and plant fresh weight (Fig. 2). As a result, the root length increased in 1.68, shoot length – in 1.7 and plant fresh weight – in 1.63 times in comparison with control.

**Fig. 2.** Cotton-plant (10 days): **A)** control, **B)** after coinoculation with the mixture of bacterial isolates HAST-2, HAST-7, HAST-9, HAST-10 and HAST-17.

Our results are agreeing with the data of earlier report (30) who reported that coinoculation of sugarcane with endophytic diazotrophs and actinomycetes significantly improved plants growth as compared to single inoculated and un-inoculated plants. It was also reported that *Lupinus angustifolius* L. seeds' inoculation with endophytic bacteria *Pseudomonas putida* L2 and *Stenotrophomonas pavanii* L8, improved plant growth and nodule formation on roots (31).

Plant beneficial traits of the isolated endophytes

The isolates HAST-2, HAST-7, HAST-9, HAST-10 and HAST-17 were tested for plant beneficial properties: nitrogen fixation, production of IAA, ACC-deaminase and siderophores and phosphates solubilization (Table 3).

The isolates possessed at least two plant growth-promoting properties. The isolates HAST-2 and HAST-7 were positive in N_2 fixation. Nitrogen is necessary for plants growth, however in the form of N_2 it is very stable and inert gas (32). Nitrogen-fixing endophytic bacteria can easily convert N_2 into ammonia which dissolves in water and directly feed a plant (33). Reports are also on isolation and characterisation of endophytic nitrogen fixing bacteria from

Table 3. Plant growth-promoting properties of the isolated endophytes

Bacterial isolates	N ₂ -fixation	IAA (µg/ml) production	Phosphates solubilization	ACC-deaminase production	Siderophores production
HAST-2	+	125.71±5.34	+	-	-
HAST-7	+	151.27±5.41	+	-	+
HAST-9	-	113.91±5.65	-	+	-
HAST-10	-	144.45±5.12	-	+	+
HAST-17	-	179.35±6.23*	+	+	+

*- statistically significant at P≤0.05 “-“ – negative, “+” – positive

sugarcane (34).

All isolates showed ability to produce IAA, however HAST-7 and HAST-17 produced higher amounts of IAA as compared to others.

Indole-3-acetic acid (IAA) is a phytohormone stimulating root growth, seed germination, takes part in metabolites biosynthesis and tolerance to various stresses (35). Endophytes producing IAA increase plant root system which in turn makes it possible to supply plant with more water and nutrients from soil (36).

The isolates HAST-2, HAST-7 and HAST-17 solubilized phosphates. Along with nitrogen, potassium is one of the most important nutrients for plant growth. Some endophytes produce organic acids which can be excreted into soil and convert phosphate complexes into orthophosphates for plant absorption and usage. Such endophytic bacteria solubilizing phosphates were suggested to use as biofertilizers (37).

The isolates HAST-9, HAST-10 and HAST-17 produced ACC-deaminase. 1-Aminocyclopropane-1-carboxylase is an ethylene precursor and the enzyme ACC-deaminase is involved in plant growth-promotion through cleavage of ACC and lowering ethylene level in plant. Ethylene is a stress hormone which leads to shortened vegetation period, defoliation and yield decrease. ACC-deaminase producing bacteria can stimulate plant growth in stress condition by lowering ethylene level (38).

The isolates HAST-7, HAST-10 and HAST-17 produced siderophores. The endophytes producing siderophores can make iron available for plant through iron chelating that is important for plants growing in iron deficient soils (37).

Biochemical characteristics of the isolated endophytes

The isolates HAST-2, HAST-7, HAST-9, HAST-10 and HAST-17 were tested for some biochemical traits (Table 4).

All tested isolates were positive in oxidase reaction, nitrate reduction, utilization of D-glucose and glycerate. None of the isolates could utilize L-rhamnose and D-xylose.

Bacterial endophytes identification

The isolates HAST-2, HAST-7, HAST-9, HAST-10 and HAST-17 were identified based on analysis of their 16S rRNA gene and matching with the closest strains from GenBank of NCBI (Table 5).

Table 4. Biochemical characteristics of the isolated endophytes

Tested properties	Bacterial isolates				
	HAST-2	HAST-7	HAST-9	HAST-10	HAST-17
Arginine dihydrolase	-	-	+	+	+
Denitrification	+	+	-	-	+
Gelatine hydrolysis	+	+	-	-	+
Starch hydrolysis	+	+	-	-	-
Levan formation	-	-	-	-	+
Lipase	+	+	-	-	+
Oxidase reaction	+	+	+	+	+
Nitrate reductase	+	+	+	+	+
Lecithinase (egg-yolk)	-	-	-	-	+
Growth at 4°C	+	-	-	-	+
41°C	+	+	-	-	-
Utilization of:					
D-Arabinose	+	+	-	-	-
L-Arabinose	+	+	-	+	-
D-Glucose	+	+	+	+	+
D-Galactose	+	+	-	-	+
Sucrose	+	+	-	-	+
L-Rhamnose	-	-	-	-	-
D-Mannose	-	-	-	+	+
Glycerate	+	+	+	+	+
D-Mannitol	+	+	-	-	+
D-Xylose	-	-	-	-	-

“-“ – negative, “+” – positive

Table 5. The effective plant growth-promoting endophytes isolated from *Halocnemum strobilaceum* (Pall.) M. Bieb. and their closest relatives from GenBank

Strain	Isolated strains deposited to GenBank		Closest match (16S rRNA genes) (GenBank)		
	Length (bp)	Accession number	Reference strains	Accession number	Percent identity
HAST-2	1467	OK594050	<i>Bacillus megaterium</i>	KY660610.1	99.93
HAST-7	1485	OK594051	<i>Bacillus aryabhatai</i>	KU179345.1	99.87
HAST-9	1427	OK594052	<i>Pseudomonas plecoglossicida</i>	MH165359.1	99.79
HAST-10	1488	OK594053	<i>Pseudomonas putida</i>	MK680517.1	99.87
HAST-17	1478	OK594054	<i>Pseudomonas chlororaphis</i>	GU947817.1	99.73

The isolates registered in GenBank as *Bacillus megaterium* HAST-2 (accession number OK594050), *Bacillus aryabhatai* HAST-7 (accession number OK594051), *Pseudomonas plecoglossicida* HAST-9 (accession number OK594052), *Pseudomonas putida* HAST-10 (accession number OK594053) and *Pseudomonas chlororaphis* HAST-17 (accession number OK594054).

Conclusion

As a result of the current research the efficient plant growth-promoting strains of the endophytic bacteria *Bacillus megaterium* HAST-2, *Bacillus aryabhatai* HAST-7, *Pseudomonas plecoglossicida* HAST-9, *Pseudomonas putida* HAST-10 and *Pseudomonas chlororaphis* HAST-17 were isolated from halophytic desert plant *Halocnemum strobilaceum* (Pall.) M. Bieb. The cotton seeds' inoculation with these

strains resulted in significant increase of seeds germination, root and shoot length, and fresh plant weight due to strains ability to fix nitrogen, produce IAA, siderophores, ACC-deaminase and solubilize phosphates. These strains can be used as bio-inoculants to improve the growth of cotton and other crops.

Authors contributions

BA and VS performed the experiments. VS analyzed data. BA statistically analyzed results. KD and BA wrote the draft of the manuscript. KD conducted the critical revision of the manuscript. ZI 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 interests to declare.

Ethical issues: None.

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