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Isolation and Characterization of Plant Growth Promoting Endophytic Bacteria from Desert Plants and Their Application as Bioinoculants for Sustainable Agriculture

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Received: 29 July 2020; Accepted: 26 August 2020; Published: 4 September 2020

Abstract: Desert plants are able to survive under harsh environmental stresses inherent to arid and semiarid regions due to their association with bacterial endophytes. However, the identity, functions, and the factors that influence the association of bacterial endophytes with desert plants are poorly known. These bacterial endophytes can be used as an untapped resource to favor plant growth and development in agro-ecosystems of arid regions. The present study is therefore focused on the isolation and identification of bacterial endophytes from two native medicinal plants (*Fagonia mollis* Delile and *Achillea fragrantissima* (Forssk) Sch. Bip.) growing spontaneously in the arid region of the South Sinai (Egypt), and characterization of their plant growth promoting (PGP) traits. Thirteen putative bacterial endophytes were isolated from the leaves of both plant species and characterized for their plant growth promoting abilities using molecular and biochemical approaches, as well as greenhouse trials. Selected endophytic bacterial strains were applied to maize plants (*Zea mays* L. var. Single cross Pioneer 30K08) to further evaluate their PGP abilities under greenhouse conditions. Isolated bacterial strains have variable plant growth promoting activities. Among these activities, isolated bacterial endophytes have the efficacy of phosphate solubilizing with clear zones ranging from 7.6 ± 0.3 to 9.6 ± 0.3 mm. Additionally, the obtained bacterial endophytes increased the productivity of indole acetic acid (IAA) in broth media from 10 to 60 $\mu g \cdot mL^{-1}$ with increasing tryptophan concentration from 1 to 5 $mg \cdot mL^{-1}$. *Bacillus* and *Brevibacillus* strains were frequently isolated from the leaves of both plant species, and had significant positive effects on plant growth and shoot phosphorus (P) and nitrogen (N) contents. Results suggest that these endophytes are good candidates as plant growth promoting inoculants to help reduce chemical input in conventional agricultural practices and increase nutrient uptake and stress resilience in plant species.

Keywords: *Zea mays* L.; environmental stresses; endophytic bacteria; plant growth promoting ability

1. Introduction

The enhancement of crop productivity is required for feeding the increasing population in developing countries and often relies on the use of chemical fertilizers. However, long-term use of these fertilizers was shown to decrease bacterial diversity in soil [1,2] and can also have harmful effects on the environment, such as leaching of phosphorus and nitrogen into groundwater, and increasing soil and groundwater pollution [3]. One way to increase the sustainability of agricultural practices is the use of efficient, nutrient mobilizing microorganisms to reduce the need and dependency on chemical fertilizers [4,5]. Plant growth promoting bacteria (PGPB) that form symbiotic interactions with their host plants are crucial to improve plant productivity and health under various environmental conditions [4,6–8]. Bacterial endophytes colonize plant tissues without any apparent pathogenic symptoms and establish beneficial associations with their plant host through phytohormone synthesis, enzyme production, and nutrient mobilization and translocation, such as phosphate (PO_4^{-3}) solubilization, nitrogen fixation, and ammonia (NH_3) production [9–11]. Moreover, many endophytes display various applications such as antimicrobial mechanisms, which reduce crop losses caused by pathogens [12–16], and its metabolites integrated into different biotechnological applications [17–20].

The Sinai Peninsula is located in the Sahara-Arabian deserts and represents approximately 6% of the total land area of Egypt. The semi-arid to arid climate and winter precipitations are the main characteristics of the Sinai Peninsula desert. Plants growing in desert conditions were found to harbor a microbiome that increased the biomass during drought stress periods [21]. Medicinal plants from desert farming in Sekem (Egypt) were shown that their roots are strongly associated with bacteria [22–24]. Although the Sinai desert has diverse medicinal plants, very few studies have focused on the associated bacterial endophytes and their PGP activities. Hanna et al. [25] collect 43 different plant species from the North Sinai desert, and reported that *Fagonia mollis* was the highest plant species harboring culturable bacteria. Among these bacteria, *Gluconacetobacter diazotrophicus* was the lowest endophytic species exhibit N_2-fixing activity. In the same regards, 132 endophytic strains were isolated from 18 Egyptian medicinal plants, including nine fungal strains isolated from *Achillea fragrantissima* and exhibiting inhibitory activities against different pathogenic bacteria and yeasts [26]. Application of the bacterial endophytes (*Bacillus thuringiensis*) led to improved plant growth and increased relative water content, chlorophyll content, chlorophyll fluorescence parameter (Fv/Fm ratio), and fruit yield of sweet pepper plants [27].

Maize has become a staple food in many parts of the world, with the total production of maize surpassing that of wheat or rice. Maize crop has several uses, such as food stuff for human or as animal feed because of its high nutritional value. Maize has also been used for corn ethanol and other maize products, such as fructose, corn starch, corn oil, and corn syrup [28]. The Arab Republic of Egypt is the largest country consuming maize at the level of the African continent. However, the production of the Arab Republic of Egypt reached to about 1% of the total global production during (2005–2013), while Egypt represents the third place at the level of the African continent [29]. Therefore, the current study aimed to improve the performance maize growth under the optimal conditions and at normal habitat.

Fagonia mollis and *Achillea fragrantissima* are medicinal plants frequently found in the Sinai Peninsula, and their bacterial endophytes could partially be responsible for the production of various bioactive compounds [30], and the ability of these plants to withstand the harsh, drought condition of the Sinai Peninsula. Therefore, this study focused on the isolation and characterization of putative bacterial endophytes from *F. mollis* and *A. fragrantissima*, which are native inhabitants of the arid and extremely harsh conditions of the Sinai desert. Plant growth promoting (PGP) properties of the bacterial endophytes involving extracellular enzymes (amylase, cellulase, protease, pectinase, and xylanase)

production, antimicrobial activity against selected pathogenic bacteria and fungi, indole-3-acetic acid (IAA) and NH_3 production, and P-solubilization ability were evaluated. In addition, their effect on maize growth, plant biomass production, and nutrients content in plant shoots were also investigated in order to evaluate their potentials as bioinoculants for sustainable agriculture practices.

2. Materials and Methods

2.1. Plant Sampling and Study Area

Fagonia mollis Delile (family *Zygophyllaceae*) and *Achillea fragrantissima* (Forssk.) Sch.Bip. (family *Asteraceae*) were collected from two sites, Wadi al-Zwatin (latitude 28.539290° to 28.53919° N, longitude 33.930784° to 33.92044° E) and Wadi Selebat (latitude 28.545493 to 28.543339 N, longitude 33.933707 to 33.932984 E), Saint Katherine Protectorate, South Sinai, Egypt (Figure 1). Four individual plants from each species were collected per site. The plant samples were carefully placed in sterile polyethylene bags and brought back to the laboratory in a portable cooler maintained at 4 °C using ice packs. The formal identification of the plant specimens was carried out at the herbarium of Botany and Microbiology Department of Al-Azhar University, where plant herbarium specimens were also deposited.

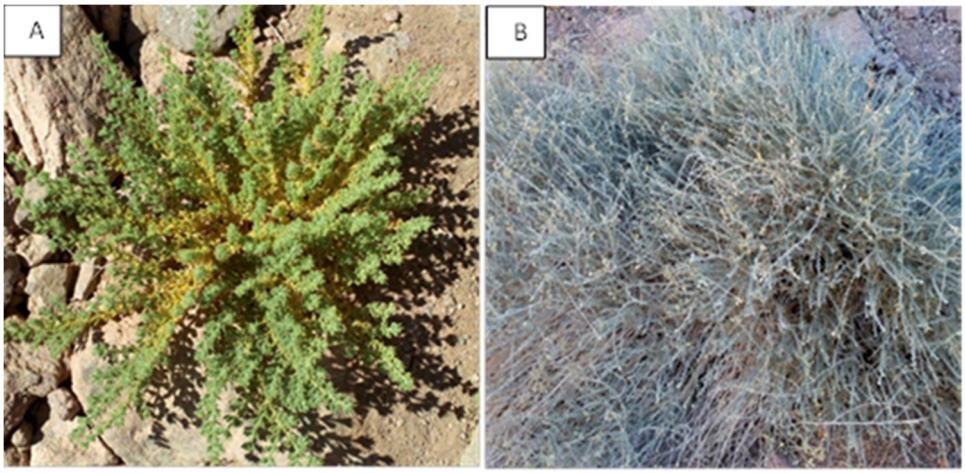

Figure 1. (**A**) *Fagonia mollis* Delile. and (**B**) *Achillea fragrantissima* Forssk.

2.2. Isolation of Bacterial Endophytes

On each plant, the first five leaves from a shoot tip were excised and washed under running tap water. Sterilization of leaf surfaces was done by soaking the tissues in a series of baths: sterile distilled water for 1 min, 70% ethanol for 1 min, 2.5% sodium hypochlorite for 4 min, 70% ethanol for 30 s, and a final series of rinsing thrice in sterile distilled water in three different containers. A 0.1 mL aliquot of the final rinse water was plated onto nutrient agar plates to confirm the success of surface sterilization.

The sterilized plant leaves were then cut into 5 mm segments, and twenty leaf segments per individual plant were placed in four petri dishes (9 cm; five segments/plate) containing luria broth (LB) media (tryptone 10 $g{\cdot}L^{-1}$; yeast extract 5 $g{\cdot}L^{-1}$; NaCl 10 $g{\cdot}L^{-1}$; agar 15 $g{\cdot}L^{-1}$; and 1 L dis. H_2O, adjusted to pH 7) supplemented with nystatin (25 $\mu g{\cdot}mL^{-1}$) to suppress fungal growth, and incubated in the dark at 35 ± 2 °C. Another twenty segments of sterilized leaves per individual plant were together crushed in 10 mL sterile saline solution using a sterile digital homogenizer (PRO25D, Pro Scentific, 120 V, Willenbrock, Oxford, CT, USA), and 1 mL of the suspension was serially diluted until 10^{-3} from which a 0.1 mL aliquot was spread onto each of the three Petri plates containing LB medium and incubated in the dark at 35 ± 2 °C [31]. The cultures were regularly observed for bacterial growth, for a period of 96 hours. Bacteria growing from the previous steps were streaked on fresh LB plates to obtain single colonies, which picked up and inoculated on LB slants and stored at 4 °C until further study.

2.3. Molecular Identification of Bacterial Endophytes

Bacterial identification was based on 16S rRNA gene sequence analysis. Genomic DNA of each isolate was extracted following the method of Miller et al. [32], with some modifications. Briefly, individual colonies from an agar plate were picked up either using a sterile toothpick or an inoculating loop and resuspended in 50 µL of sterile deionized water. The cell suspension was placed in a water bath at 97 °C and heated for 10 min, the cell lysate was centrifuged (15,000× *g*, 10 min), and the supernatant containing the DNA was recovered. DNA concentration was determined by measuring its absorbance at UV spectrum of 260 nm using a spectrophotometer (JENWAY 6350, 230 V/50 Hz, Staffordshire, UK). A partial 16S rDNA fragment was PCR amplified using the bacterial universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) [33]. The PCR reaction contained: 1 × PCR buffer, 0.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase (QIAGEN), 0.25 mM dNTP, 0.5 µM of each primer, and approximately 5 ng of bacterial genomic DNA. The PCR cycling conditions were 94 °C for 3 min, followed by 30 cycles of 94 °C for 0.5 min, 55 °C for 0.5 min, and 72 °C for 1 min, followed by a final extension performed at 72 °C for 10 min. The PCR products were forward and reverse sequenced using the Applied Biosystems 3730xl DNA Analyzer technology at the Genome Quebec Innovation Center (Montreal, QC, Canada). The sequences generated in this study were deposited in GenBank under accession numbers KY555785 to KY555797. The 16S rRNA sequences were then compared against the GenBank database using the NCBI BLAST nucleotide search. A multiple sequence alignment was constructed on approximately 1200 bp of 16S rRNA gene fragments using the ClustalX 1.8 software package (http://www.clustal.org/clustal2) and a phylogenetic tree was constructed using the neighbor-joining method in the MEGA v6.1 software (www.megasoftware.net), with confidence tested by bootstrap analysis (1000 repeats).

2.4. Screening the Extracellular Enzymatic Activities of Bacterial Endophytes

The production and activity of extracellular enzymes (amylase, cellulase, protease, pectinase, and xylanase) of isolated bacterial endophytes were assessed by growing the isolates in a mineral salt (MS) media ($NaNO_3$ 5 g·L^{-1}; KH_2PO_4 1 g·L^{-1}; K_2HPO_4 2 g·L^{-1}; $MgSO_4.7H_2O$ 0.5 g·L^{-1}; KCl 0.1 g·L^{-1}; $CaCl_2$ 0,01 g·L^{-1}; $FeSO_4.7H_2O$ 0.02 g·L^{-1}; agar 15 g·L^{-1}; and 1 L dis. H_2O) complemented with various additives, depending on the enzyme being tested, as detailed below. Control treatments consisted of the same media without bacterial inoculation. After incubation for 24–48 h depending on the growth rates of the bacterial endophytes at 35 ± 2 °C, specific reagents were added (see paragraph below), and the size of the clear zone surrounding the bacterial colony was measured, indicating extracellular enzymatic activities. All assays were performed in triplicates.

Amylolytic and cellulase activity were assessed by growing the endophytic bacterial isolates on MS agar medium supplemented with 1% soluble starch and 1% cellulose or carboxy-methylcellulose (CMC) respectively. After incubation, the plates were flooded with 1% iodine. MS agar medium containing 1% gelatine was used to determine the bacterial proteolytic activity. After incubation, the degradation of gelatine was highlighted using acidic mercuric chloride as an indicator. Pectinolytic activity was determined by growing bacteria in MS medium containing 1% pectin. After the incubation period, the plates were flooded with 1% aqueous solution of hexadecyl trimethyl ammonium bromide. MS agar medium supplemented with 1% xylan from corncobs was used to measure bacterial xylanolytic activity. After the incubation period, the xylanase activity was assessed after flooding with absolute ethyl alcohol to indicate biodegradation [34].

2.5. Antimicrobial Activity of Bacterial Endophytes

To test the antimicrobial activity of the bacterial endophytes, the isolated strains were cultured in nutrient broth medium for 6 days at 35 ± 2 °C on a shaker (LABOAO, LH-2102C, Zhengzhou, China) at 180 rpm. Crude fermentation broth was blended thoroughly and centrifuged at 4000 rpm for 5 min. Liquid supernatant was extracted twice with an equal volume of ethyl acetate. The organic solvent

extract was then evaporated under reduced pressure using a rotary evaporator (RE-801, BM-500 water bath (4 L), glassware C set, Yamato scientific, Tokyo, Japan). The crude extracts were dissolved in dimethyl sulfoxide (DMSO) and used for antimicrobial screening using a well diffusion method [35]. Nutrient broth media without bacterial inoculation were extracted and dissolved in DMSO and were used as controls.

Microbial strains used for antimicrobial assays were: *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633 (Gram-positive bacteria), *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella typhimurium* ATCC 14028 (Gram-negative bacteria), and *Candida albicans* ATCC 10231 (yeast). Test organisms were inoculated in Petri dishes containing Muller–Hinton agar medium (Sigma-Aldrich) for bacteria or Sabouraud agar medium (Sigma-Aldrich) for yeast [19,36,37]. Three wells of 1 cm diameter were cut in the tested organism colony using a sterile cork borer and filled with 40 µL of endophytic bacterial extract. Negative control wells were filled with 40 µL of control extract. The plates were kept at 4 °C for 4 h to allow diffusion of antimicrobial compounds, and then incubated at 35 ± 2 °C for bacteria and 28 ± 2 °C for *C. albicans* for 24 h [38,39]. The inhibition zones around the wells were measured to assess the antimicrobial activity of bacterial extracts. All antimicrobial activity assays were performed in triplicates.

2.6. Screening for In Vitro Plant Growth Promoting (PGP) Traits

2.6.1. Phosphate Solubilization

The bacterial endophytic isolates were screened for P-solubilization as follows. Pikovskaya medium (glucose 10 g·L^{-1}; $Ca_3(PO_4)$ 2·5 g·L^{-1}; $(NH_4)2SO_4$ 0.5 g·L^{-1}; NaCl 0.2 g·L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.1 g·L^{-1}; KCl 0.2 g·L^{-1}; $FeSO_4 \cdot 7H_2O$ 0.002 g·L^{-1}; yeast extract 0.5 g·L^{-1}; $MnSO_4 \cdot 2H_2O$ 0.002 g·L^{-1}; agar 15 g·L^{-1}; and 1 L dis. H_2O) was prepared and bromophenol blue was added as an indicator. The medium was inoculated with endophytic isolates and incubated for 48 h. The Pikovskaya medium without bacterial growth was used as a control. The formation of clear zones around the colony, due to the utilization of tricalcium phosphate, was measured to assess the ability of endophytes to solubilize phosphate [40].

2.6.2. Ammonia Production

The ability of the isolated endophytic bacterial strains to produce NH_3 was assessed after growing the bacterial strains in peptone water (peptone 10 g·L^{-1}; NaCl 5 g·L^{-1}; and 1 L dis. H_2O) for 72 h at 35 ± 2 °C. Peptone water without bacterial inoculation was used as a control. The addition of 1 mL of Nessler's reagent in the peptone liquid medium was used to assess the ammonia production. A color change to faint yellow indicated the minimum ammonia production while deep yellow to brownish color indicated the maximum ammonia production [41].

2.7. Quantitative Screening for Indole-3-acetic acid (IAA) Production

The ability of bacterial endophytes to produce IAA was determined in nutrient broth at 35 ± 2 °C for 24 h. One milliliter of each bacterial suspension was added to 20 mL of nutrient broth medium containing 0, 1, 2, or 5 mg·mL^{-1} tryptophan, and incubated for 14 days. Controls consisted of nutrient broth media containing 0, 1, 2, or 5 mg·mL^{-1} tryptophan but without bacterial inoculation. Five milliliters of each culture were collected from the incubating broth after 14 days and centrifuged at 6000 rpm for 30 min. One milliliter of the supernatant was mixed with 1 drop of orthophosphoric acid and 2 mL of Salkowski's reagent (300 mL concentrated sulfuric acid, 500 mL distilled water, and 15 mL 0.5 M $FeCl_3$). Development of a pink color indicated IAA production. The optical density at 530 nm was measured using a spectrophotometer (Jenway 6305 UV spectrophotometer, 230 V/50 Hz, Staffordshire, UK), and the amount of IAA produced was estimated using a standard curve for authentic IAA [42].

Five isolates were chosen based on their ability to produce IAA for further analysis. Equal amount of each inoculum was added to nutrient broth medium containing 5 mg mL^{-1} tryptophan and incubated

for 14 days at 35 ± 2 °C. IAA concentration was determined at 2 days intervals up to the 14th day after inoculation. Samples were centrifuged at 6000 rpm for 30 min and IAA production was determined as mentioned above. All the IAA production assays were performed in triplicates.

2.8. Effect of Bacterial Isolates on Zea mays L. Growth

2.8.1. Experimental Design

A pot experiment was conducted in a completely randomized design with five replicates of each treatment. Plants were inoculated with one of five individual bacterial isolates (*Brevibacillus* spp. Af.13, and Af.14, *Bacillus* spp. Fm.3, Fm.4, and Fm.6) or with a bacterial consortium formed of an equal amount of the five bacteria isolates. A control treatment consisted of uninoculated plants.

2.8.2. Culture Conditions

A loamy soil was collected from an agricultural field in the El-Menoufia governorate. Physical and chemical characteristics of the soil are shown in Table 1. The soil was air-dried, sieved with a 2 mm sieve, mixed with quartz sand at a soil: sand ratio of 3:1 and autoclaved twice for one hour at 121 °C. The five most potent IAA producing bacterial isolates (as listed above) were inoculated in nutrient broth and incubated at 35 ± 2 °C for 24 h on a shaker (LABOAO, LH-2102C, Zhingzhou, China) at 180 rpm. Seeds of maize (*Zea mays*, Cultivar Giza 9) were surface sterilized by soaking in 2.5% sodium hypochlorite for 3 minutes and then washed 5 times in sterile distilled water. Six groups of pregerminated seeds were separately incubated in 50 mL aliquots of the culture medium inoculated each with one of the five bacterial strains or with the bacterial consortium, and incubated at room temperature for 4 h on a shaker at 180 rpm. After incubation, the soaked seeds were sown in 1 L plastic pots filled with 900 g of sterilized soil-sand mixture. Each pot received three germinated seeds. Plants were grown in a greenhouse with a temperature of 25–30 °C and were irrigated with tap water as required without fertilization.

Table 1. Physical and chemical characteristics of the soil used in the greenhouse experiment.

Parameters	Soil Analysis
Soil Texture	Loamy Sand
Physical characters (%)	
Sand	76.8
Silt	10.9
Clay	12.2
Chemical characters (mg kg^{-1})	
P	24
K	14.075
Na	186.44
Ca	27.25
Cl	134.35

2.9. Plant Tissue Analysis

After 30 days, plants were harvested, shoot and root systems were separated, and roots were washed carefully with tap water to remove the attached soil particles. The dry weight of shoots and roots were measured after drying for 48 h at 60 °C. Phosphorus, nitrogen, and potassium contents were determined according to the methods described by AOAC international [43] and Rice et al. [44].

2.10. Statistical Analysis

Data were statistically analyzed using SPSS v17 (SPSS Inc., Chicago, IL, USA). When the normality and homogeneity of variance hypotheses were satisfied, one-way analysis of variance (ANOVA) was

used to compare the bacterial isolates for extracellular enzymes production, antimicrobial activity, IAA and ammonia production, P-solubilization ability, and the effect of these endophytes on maize growth performance. A posteriori multiple comparisons were done using Tukey's range tests at $p < 0.05$. All results are the means of three to five independent replicates, as specified above.

3. Results

Thirteen endophytic bacterial strains were isolated from the leaves of the two medicinal plants (Table 2). Nine strains were isolated from *F. mollis* plants and identified as *Bacillus* spp. (eight strains), and *Paenibacillus* sp. (one strain), while four bacterial strains were isolated from *A. fragrantissima* plants and identified as *Paenibacillus* sp. (one strain) and *Brevibacillus* sp. (three strains). The 16S rRNA gene sequences of strains Fm.2 to Fm.9 showed 96–99% sequence similarity with the sequences of *Bacillus amyloliquefaciens*, *Bacillus thuringiensis*, and *Bacillus cereus* (Figure 2 and Table 2). The 16S rRNA gene sequences of strains Fm.1 and Af.12 showed 99% of sequence similarity with *Paenibacillus barengoltzii*. Isolates Af.13 to Af.15 showed between 93 and 99% of 16S rRNA sequence similarity with *Brevibacillus agri*.

All bacterial endophytes isolated from *F. mollis* were positive for amylase, pectinase, carboxymethyl cellulase, cellulose, xylanase, and gelatinase, while those isolated from *A. fragrantissima* showed activities for only one to four enzymes (Table 3). The highest activities of cellulase and carboxymethyl cellulase were observed with *Bacillus* sp. Fm.5 (22.0 ± 1.1 and 21.3 ± 1.2 mm, respectively), while *Bacillus* sp. Fm.2 showed the highest activities of pectinase, xylanase, and gelatinase enzymes with clear zone 17.6 ± 0.6, 19.6 ± 0.3, and 22.0 ± 0.5 mm respectively. The highest gelatinase activity (22.3 ± 1.4 mm) was measured for *Bacillus* sp. Fm.3.

The antimicrobial activity of the bacterial endophytes against selected pathogenic bacterial and yeast strains are given in Table 4. The crude extract of *Brevibacillus* sp. Af.13 suppressed the growth of five tested pathogenic microorganisms, while *Bacillus* sp. Fm.8 inhibited the growth of *P. aeruginosa*, *S. typhi*, and *E. coli*. Endophytic strains *Bacillus* sp. Fm.2 and *Brevibacillus* sp. Af.13 were the only endophytes whose crude extracts showed an inhibitory effect against the pathogenic yeast *C. albicans* ATCC 10231 with clear zone 15 and 18 mm respectively. While the filtrates extracted from all strains showed some inhibition of *P. aeruginosa* ATCC 9027, the highest growth inhibition was noted from strains Fm.6, Fm.7, Fm.8, Fm.9, and Af.14 with inhibition zones ranging between 15 to 30 mm.

Table 2. The 16S rRNA sequence identification of endophytic bacterial strains from two different medicinal plants.

Plant Species	Bacterial Strain Code	Homologue Sequences (Sequence Identity %)	NCBI Accession Numbers
Fagonia mollis	Fm.1	*Paenibacillus barengoltzii* (99%)	NR_042756
	Fm.2	*Bacillus amyloliquefaciens* (98%)	NR_117946
	Fm.3	*Bacillus thuringiensis* (97%)	NR_043403
	Fm.4	*Bacillus cereus* (98%)	NR_115526
	Fm.5	*Bacillus cereus* (99%)	NR_115526
	Fm.6	*Bacillus thuringiensis* (98%)	NR_114581
	Fm.7	*Bacillus amyloliquefaciens* (97%)	NR_117946
	Fm.8	*Bacillus cereus* (97%)	NR_115526
	Fm.9	*Bacillus cereus* (96%)	NR_115526
Achillea fragrantissima	Af.12	*Paenibacillus barengoltzii* (99%)	NR_113988
	Af.13	*Brevibacillus agri* (95%)	NR_113767
	Af.14	*Brevibacillus agri* (93%)	NR_113767
	Af.15	*Brevibacillus agri* (99%)	NR_113767

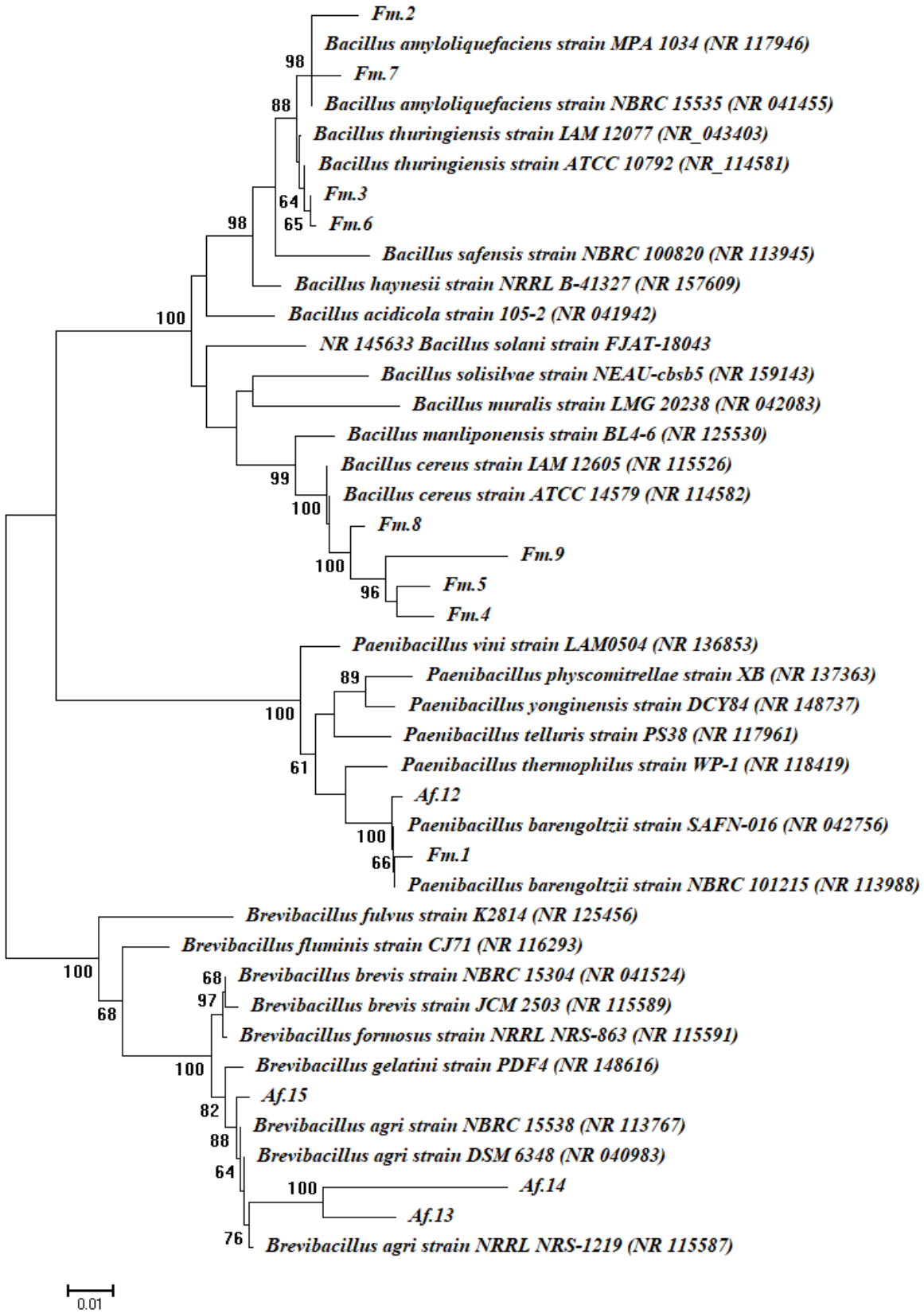

Figure 2. Phylogenetic analysis of 16S rRNA sequences of bacterial strains with reference sequences from NCBI. Fm.1–Fm.9 refers to 16S rRNA sequences of bacteria isolated from *Fagonia mollis* plants, whereas Af.13–Af.15 are the sequences from isolates from *Achillea fragrantissima*. Identity of the bacterial isolates is available in Table 2. The analysis was performed in MEGA 6 using the neighbor-joining method.

Table 3. Extracellular enzymatic activities of bacterial endophytes.

Bacterial Strains [1]	Diameter of Clear Zones (mm) [2]					
	Amylase	Pectinase	CMCase [3]	Cellulase	Xylanase	Gelatinase
C	0 ^{d}	0 ^{d}	0 ^{e}	0 ^{d}	0 ^{f}	0 ^{f}
Fm.1	17.6 ± 1.2 ^{a}	16.3 ± 0.3 ^{b}	18.6 ± 0.6 ^{b}	20.0 ± 1.7 ^{b}	17.0 ± 1.0 ^{b}	21.0 ± 0.5 ^{a}
Fm.2	17.3 ± 0.8 ^{a}	17.6 ± 0.6 ^{a}	20.6 ± 0.6 ^{a}	18.6 ± 0.8 ^{c}	19.6 ± 0.3 ^{a}	22.0 ± 0.5 ^{a}
Fm.3	17.0 ± 1.5 ^{a}	17.0 ± 0.5 ^{a}	18.0 ± 1.0 ^{b}	18.6 ± 1.2 ^{c}	16.0 ± 00.0 ^{b}	22.3 ± 1.4 ^{a}
Fm.4	17.6 ± 0.8 ^{a}	17.0 ± 0.5 ^{a}	19.0 ± 0.5 ^{b}	19.6 ± 1.7 ^{b}	14.6 ± 0.3 ^{c}	20.6 ± 0.6 ^{b}
Fm.5	17.6 ± 0.8 ^{a}	16.3 ± 0.8 ^{b}	21.3 ± 1.2 ^{a}	22.0 ± 1.1 ^{a}	18.6 ± 0.3 ^{a}	21.6 ± 0.8 ^{a}
Fm.6	17.0 ± 1.1 ^{a}	15.6 ± 0.3 ^{c}	18.3 ± 0.8 ^{b}	16.3 ± 0.3^{d}	16.3 ± 0.8^{b}	17.0 ± 1.5 ^{d}
Fm.7	17.3 ± 0.6 ^{a}	17.0 ± 0.5 ^{a}	15.6 ± 0.8 ^{c}	16.6 ± 1.2 ^{d}	14.3 ± 0.6 ^{c}	15.0 ± 1.5 ^{e}
Fm.8	17.6 ± 0.6 ^{a}	14.6 ± 0.3 ^{c}	18.0 ± 1.15 ^{b}	18.3 ± 0.3 ^{c}	14.3 ± 0.6 ^{c}	18.3 ± 1.6 ^{c}
Fm.9	15.3 ± 1.7 ^{b}	15.6 ± 0.3 ^{c}	18.6 ± 0.3 ^{b}	17.6 ± 0.8 ^{c}	9.0 ± 1.1 ^{e}	18.0 ± 1.5 ^{c}
Af.12	9.3 ± 0.3 ^{c}	0 ^{d}	19.3 ± 0.3 ^{b}	20.6 ± 0.6 ^{b}	13.6 ± 0.3 ^{d}	0 ^{f}
Af.13	0 ^{d}	0 ^{d}	0 ^{e}	0 ^{d}	0 ^{f}	19.0 ± 1.5 ^{c}
Af.14	0 ^{d}	14.0 ± 0.2 ^{d}	0 ^{e}	0 ^{d}	0 ^{f}	0 ^{f}
Af.15	0 ^{d}	17.6 ± 0.3 ^{a}	11.0 ± 1.0 ^{d}	17.33 ± 1.76 ^{c}	0 ^{f}	20.3 ± 2.3 ^{b}

[1] C: controls without bacterial inoculation. Identity of the bacterial isolates is available in Table 2. [2] Different letters between lines denote that mean values are significantly different ($p \leq 0.05$) by Tukey's test, means ± Standard Error (SE) ($n = 3$). [3] Carboxymethyl cellulase.

Table 4. Antimicrobial activities of bacterial endophytes.

Bacterial Strains [1]	Diameter of Clear Zone (mm)					
	P. aeruginosa	*S. typhi*	*E. coli*	*S. aureus*	*B. subtilis*	*C. albicans*
C	–	–	–	–	–	–
Fm.1	17	–	–	–	–	–
Fm.2	15	–	–	–	–	15
Fm.3	15	–	–	–	–	–
Fm.4	15	–	–	–	–	–
Fm.5	17	–	–	–	–	–
Fm.6	20	–	–	–	–	–
Fm.7	22	–	18	–	–	–
Fm.8	30	15	18	–	–	–
Fm.9	25	–	20	–	–	–
Af.12	15	–	–	–	–	–
Af.13	15	11	11	–	17	18
Af.14	20	–	–	–	–	–
Af.15	15	–	–	–	–	–

[1] C: controls without bacterial inoculation. Identity of the bacterial isolates is available in Table 2.

All endophytes identified as *Brevibacillus* sp. produced the highest amount of ammonia compared to *Bacillus* spp. strains (Table 5). Moreover, nine endophytes (Fm.2 to Fm.9 and Af.12) displayed significant ability to solubilize inorganic phosphate with clear zone on the Pikovskaya medium ranging from 7.6 ± 0.3 to 9.6 ± 0.3 mm. Results showed that all the isolated strains were IAA producers, with or without tryptophan (Figure 3). However, increasing tryptophan concentration from 1 to 5 mg·mL^{-1} resulted in increased bacterial ability to produce IAA from 10 to 60 µg·mL^{-1}. Strains of *Brevibacillus* spp. Af.14, Af.13, *Bacillus* sp. Fm.6, *Bacillus* sp. Fm.4, and *Bacillus* sp. Fm.3 produced the highest amount of IAA, and were selected for further analysis to measure the production of IAA at 2 day intervals in a time course over 14 days. The results indicated that the maximum IAA production with tryptophan was 5 mg mL^{-1} after 10 days. The results revealed that *Brevibacillus* sp. Af.14 produced the highest amount of IAA 59.7 µg·mL^{-1} ($p \leq 0.05$; Figure 4).

Table 5. Ammonia production and phosphate solubilization of endophytic bacterial strains.

Bacterial Strains [1]	Ammonia Production [2]	*P solubilization* Diameter of Clear Zone (mm) [3]
C	-	0 [d]
Fm.1	–	0 [d]
Fm.2	+	8.6 ± 0.3 [b]
Fm.3	++	9.0 ± 0 [a]
Fm.4	-	7.6 ± 0.3 [c]
Fm.5	++	9.6 ± 0.3 [a]
Fm.6	+	8.6 ± 0.3 [b]
Fm.7	+	8.3 ± 0.3 [b]
Fm.8	-	9.3 ± 0.3 [a]
Fm.9	++	9.3 ± 0.3 [a]
Af.12	++	9.3 ± 0.3 [a]
Af.13	++	0 [d]
Af.14	++	0 [d]
Af.15	++	0 [d]

[1] C: controls without bacterial inoculation. Identity of the bacterial isolates is available in Table 2. [2] -, +, and ++ denote no, low, and high ammonia production, respectively. [3] Different letters between columns denote that mean values are significantly different ($p \leq 0.05$) by Tukey's test, means ± SE ($n = 3$).

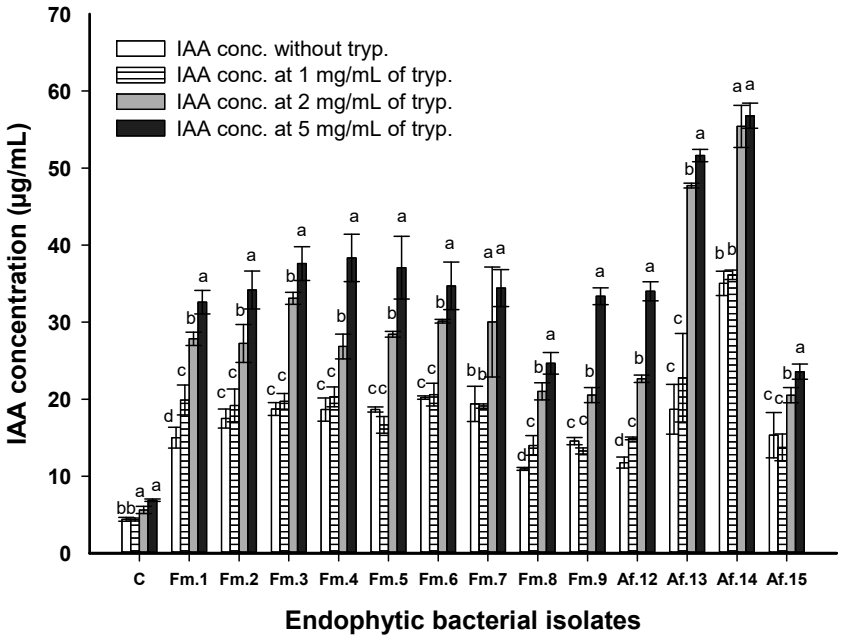

Figure 3. Quantitative production of IAA by endophytic bacterial strains with and without tryptophan. C, controls without bacterial inoculation. Identity of the bacterial isolates are available in Table 2. Data are statistically different at $p \leq 0.05$ by Tukey's test, ($n = 3$); error bars are means ± SE. Bars with the same letter for each endophytic isolate did not differ significantly, different letters on bars denote that mean values are significantly different at significant level of ($P \leq 0.05$), error bars are means ± SE.

In the greenhouse experiment, all maize plants inoculated with bacterial endophytes yielded significantly higher dry shoot weights ($F_{6,28} = 11.09$ and 10.33 respectively; $p \leq 0.001$) compared to the uninoculated control plants (Table 6). Plants inoculated with bacterial endophytes produced dry root weight higher than those recorded in control plants, but the differences were not significant ($F_{6,28} = 1.51$; $p = 0.21$).

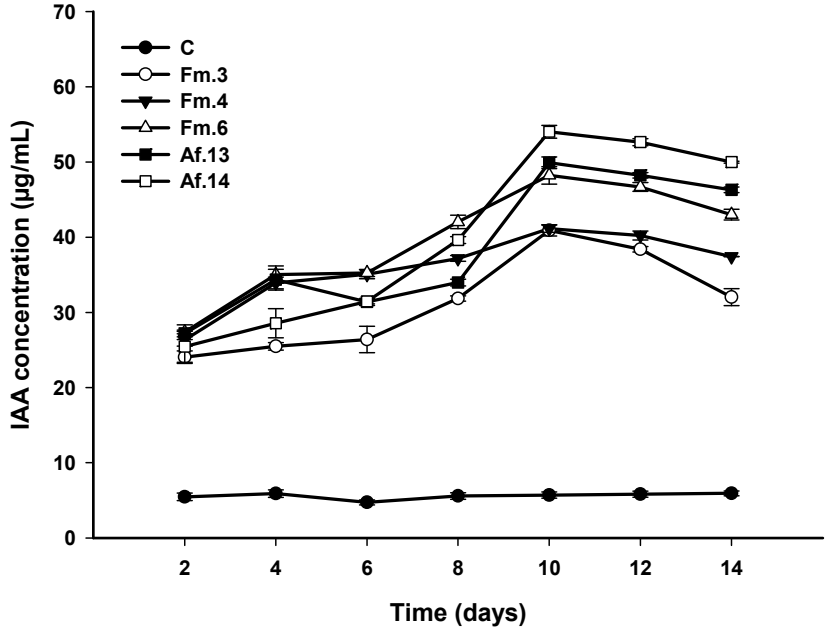

Figure 4. IAA production by the most potent bacterial strains in the presence of 5 mg mL^{-1} tryptophan and over 14 days. C, controls without any bacterial inoculation. Identity of the bacterial isolates is available in Table 2. At each time point, bars with the same letter did not differ significantly at a significant level of ($p \leq 0.05$) by Tukey's test, ($n = 3$).

Table 6. Effect of bacterial inoculations on the growth properties of maize plants.

Bacterial Strains [1]	Dry Weight (mg) [2]		Shoot Nutrients Content (mg)		
	Shoot	Root	P	N	K
C	82 ± 3.56 ^{c}	252.8 ± 19.5 ^{a}	0.42 ± 0.01 ^{c}	2.2 ± 0.17 ^{c}	8.70 ± 0.03 ^{b}
Fm.3	110.3 ± 5.5 ^{a}	322.64 ± 16.0 ^{a}	1.05 ± 0.07 ^{a}	3.9 ± 0.19 ^{b}	11.35 ± 0.92 ^{a}
Fm.4	81.8 ± 4 ^{c}	315.8 ± 27.9 ^{a}	0.72 ± 0.03 ^{b}	3.1 ± 0.60 ^{bc}	8.80 ± 0.62 ^{b}
Fm.6	103.2 ± 1.8 ^{a}	295.66 ± 30.1 ^{a}	0.76 ± 0.02 ^{b}	4.4 ± 0.18 ^{b}	11.11 ± 0.31 ^{a}
Af.13	108.2 ± 1.05 ^{a}	286.86 ± 19.3 ^{a}	0.44 ± 0.01 ^{c}	7.1 ± 0.18 ^{a}	10.57 ± 0.60 ^{ab}
Af.14	90.78 ± 4.55 ^{b}	303.8 ± 27.3 ^{a}	0.40 ± 0.02 ^{c}	7.2 ± 0.43 ^{a}	9.97 ± 0.13 ^{ab}
Mix	95.2 ± 2.8 ^{b}	249.6 ± 19.7 ^{a}	0.40 ± 0.01 ^{c}	3.4 ± 0.27 ^{bc}	10.45 ± 0.36 ^{ab}

[1] C: controls without bacterial inoculation. Identity of the bacterial isolates is available in Table 2. Mix, bacterial consortium consists of Fm.3, Fm.4, Fm.6, Af.13, and Af.14. [2] Different letters between columns denote that mean values are significantly different ($p \leq 0.05$) by Tukey's test, means ± SE ($n = 5$).

Inoculation of maize plants with *Bacillus* spp. Fm.3, Fm.4, and Fm.6 significantly ($F_{6,14} = 49.07$; $p \leq 0.001$) increased P shoot contents (1.05 ± 0.07, 0.72 ± 0.03, and 0.76 ± 0.02 mg respectively) as compared to the un-inoculated control plants (0.42 ± 0.01 mg), while *Brevibacillus* spp. Af.13, Af.14, and the bacterial consortium formed by a mixture of the five isolates did not affect shoot P content compared to control plants (Table 6). Analysis showed that bacterial inoculation significantly increased N shoot contents compared to the control plants ($F_{6,14} = 35.76$, $p \leq 0.001$; Table 6). Plants inoculated with *Bacillus* spp. Fm.3, and Fm.6, *Brevibacillus* spp. Af.13, and Af.14 had significantly higher ($p \leq 0.05$) N contents (range of 3.9 ± 0.19 to 7.2 ± 0.43 mg) than those treated with other strains or uninoculated. The bacterial strains *Bacillus* spp. Fm.3 and Fm.6 significantly ($F_{6,14} = 4.15$; $p = 0.013$) increased K shoot contents (11.35 ± 0.92 and 11.11 ± 0.31 mg) in comparison with uninoculated control plants (8.70 ± 0.03 mg).

4. Discussion

In this study, 13 putative bacterial endophytic strains were isolated from two medicinal plants growing under the adverse conditions of the Sinai desert. Nine bacterial endophytes were isolated

from *F. mollis* and identified as different species of *Bacillus*, and *Paenibacillus*, and four bacterial endophytes were isolated from *A. fragrantissima* and identified as *Paenibacillus* spp. and *Brevibacillus* spp. (Table 2). The plant growth promoting (PGP) activities of these bacterial strains were characterized, including extracellular enzyme production, antimicrobial action, IAA and ammonia production, and P-solubilization. In the same regard, Eida et al. [45] reported isolation of endosphere and rhizosphere bacterial groups associated with four native Saudi desert plants and proved their plant growth promotion potential including phosphate solubilization and IAA production. Based on PGP characteristics, five endophytic bacterial strains were selected to evaluate their effects on plant growth and development. Results showed that the selected endophytes have key PGP properties, and significantly increased dry weight of tissues, and P concentrations in shoots of maize plants compared with uninoculated controls. Corresponding with our results, Marag and Suman [42] isolated six bacterial endophytes including *Bacillus cereus* from two cultivars of maize, and the pot experiment indicates the efficacy of the isolates in improving biomass parameters of inoculated maize plants, in addition to compensating for approximately 25% of the NPK fertilizer input.

The bacterial endophytes exhibited different enzymatic activities involving cellulase, pectinase, xylanase, amylase, and gelatinase production [46,47]. Cellulolytic and pectinolytic activities are known to enable microorganisms to penetrate plant tissues and establish a symbiotic relationship with their host plants. The *Bacillus* spp. strain isolated in this study showed high hydrolytic activity for cellulose and pectin, as well as proteolytic activity. Similarly, different endophytic strains of *Bacillus* were shown to be strong producers of cellulase and pectinase [48]. The extracellular hydrolytic enzymes produced by endophytes contribute indirectly to plant growth promotion and protection against pathogens [49,50]. The endophytes can be described as bioproducers for amylases and xylanases based on their amylolytic and xylanolytic activities. Similarly, bacterial endophytes isolated from mangrove plants had activities associated with amylases [50]. The diverse enzymatic activities of the isolated endophytes showed their capability to catalyze different biochemical reactions and their potential for agricultural and industrial applications. Likewise, Castro et al (2014) isolated endophytic *Bacillus* from two Brazilian mangrove species, the isolates displayed extracellular amylase, esterase, lipase, protease, and endoglucanase activities and thus can be used in industrial applications [50]. Moreover, theses enzymes could enable endophytes to penetrate plant tissues and build a symbiotic relationship with their host plant, besides protecting the host from pathogens by hydrolysis of the pathogen cell wall [10].

Antimicrobial activities of the isolated endophytes were evaluated based on the suppression of microbial growth caused by the crude extracts. The estimation of antimicrobial activity of crude extracts is the initial step required for the discovery of new antimicrobial compounds. Selection of bacterial isolates as inoculants based on their PGP traits, and on their inhibitory effect against different pathogens, has received attention and has been suggested as an approach to enhance plant growth and protect plants against diseases [51]. In the current study, the isolated endophytes showed a significant antagonistic effect against different pathogenic microorganisms. Endophytic bacteria can indirectly assist plant growth through the production of substances, which inhibit plant pathogens [52,53]. Endophytes isolated from other medicinal plants have also produced novel bioactive compounds [49,54]. Hassan [10] isolated six bacterial endophytes including *Bacillus cereus* and *Bacillus subtilis* from the native desert medicinal plant *Teucrium polium* L., the isolates manifested variable broad-spectrum activity against *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Candida albicans*. Accordingly, suggesting their application as biocontrol agents [10].

Sun et al. [55] showed that 10 endophytic bacterial strains of *Bacillus* and *Streptomyces* isolated from *Polygonum cuspidatum* exhibited antagonistic effects against different plant pathogens. *Bacillus licheniformis* and *Bacillus pumilus* endophytic isolates from *Platycodon grandiflorum* roots also exhibited a significant antifungal action against *Phytophthora capsici*, *Fusarium oxysporum*, *Rhizoctonia solanic*, and *Pythium ultimum*. The endophytic bacterial strain *Paenibacillus* sp. IIRAC-30 was

isolated from cassava and suppressed the growth of *Rhizoctonia solani* [56]. *Bacillus amyloliquefaciens* was isolated from the Chinese medicinal plant *Scutellaria baicalensis* Georgi. A crude extract of this strain exhibited antagonistic effects against some plant pathogens, food-borne pathogenic and spoilage microorganisms [53].

The PGP properties of bacteria have been investigated to select bacteria with high potential to be used as biofertilizers. These tests are critical in light of the fact that they identify bacteria with higher benefits for plants before testing them in field traits [57]. Ammonia and IAA production, as well P-solubilization, are among various mechanisms exhibited by bacteria that enhance plant growth [58]. Here, most endophytic bacterial isolates were able to produce different amounts of ammonia. It is often found that ammonia-producing bacteria can supply ammonia as a nitrogen source for plant growth [59]. Bacterial endophytes can enhance plant growth through the production of ammonia through the hydrolysis of urea into ammonia and carbon dioxide [60]. With regard to P-solubilization, most of the isolated endophytes showed variable capacity to solubilize phosphate. Rodrigues et al. [61] found that about 47% of bacterial endophytes isolated from sugarcane have low P-solubilizing indices. At soil with low phosphate supply, inoculation of P-solubilizing endophytic bacteria leading to increase of pant growth performance.

Indole-3-acetic acid (IAA) is a phytohormone that can be produced by plants and various microorganisms. This hormone not only enhances plant growth but also contributes in the interaction between plants and microorganisms [62]. In this study, all endophytic bacterial strains had the ability to produce IAA in the absence and presence of tryptophan, the precursor for IAA production. Although most microorganisms utilize tryptophan in IAA synthesis [63,64], the advantage of bacterial endophytes is that they can produce IAA without tryptophan supplementation. Rodrigues et al. [61] showed that 57% of bacterial endophytes secreted high IAA concentration of 21.05–139.21 $\mu g\ mL^{-1}$ in 72 h in the presence of 5 mM tryptophan. Endophytic bacterial strains were shown to produce higher IAA concentrations than rhizospheric strains, suggesting a closer link, and potential symbiosis, between endophytes and their hosts [12]. Thus, in the current study, a higher capacity to produce IAA was used to select five bacterial strains to determine their effect on maize growth performance. Bokhari et al. [65] isolated *Bacillus circulans* PK3-138 from plants grown in Pakistan desert, reported the potency of this isolate for IAA production. Similarly, four bacterial endophytes (*Sphingomonas* sp., *Bacillus* sp., *Pantoea* sp., and *Enterobacterc* sp.) isolated from the roots of elephant grass showed valuable PGP traits including IAA production at a range of 10.50–759.19 mg/L, and ammonia production capacity. So, these inoculants could be used for increasing crop yield in a sustainable mode [58].

We found that inoculated plants produced more biomass than uninoculated plants. Plant–microbe interactions are well known to influence nutrient transfer between microorganisms and plants [66]. Therefore, it is possible that plant biomass production varied with different microbial taxa assemblages in the roots due to their various abilities to supply nutrients to their host. The results showed that the shoot P concentration was significantly increased in plants inoculated with Fm.3, Fm.4, and Fm.6 compared to the uninoculated plants. P-solubilizing bacteria help plants to access insoluble forms of phosphate, such as apatite, through excretion of protons and organic acids, mainly gluconic acid, rendering phosphate available to plants for uptake [11,67]. These bacteria can also produce enzymes that mineralize organic phosphorus, which also render it available for plants [67]. The capacity of microorganisms to absorb immobile nutrients such as P from soils and transfer it to their host plants is one of the main effects of microbial symbiosis; however, microbial capacity for nutrient transfer varies with different microorganisms [68]. Basically, plant roots can be colonized simultaneously by multiple microorganisms, which can positively or negatively benefit the host plant [69,70].

Importantly, not only did the isolated endophytic *Bacillus* and *Brevibacillus* species display the highest level of IAA and ammonia production, but they also had various plant growth-promoting traits. Bacteria that were isolated and characterized in the present study are potential candidates for plant bioinoculation in agricultural practices, in particular those that inhibited pathogens and harbored the highest levels of IAA production and of nutrient uptake.

5. Conclusions

Very few isolates were obtained herein to claim that, bacterial endophytes inhabiting the two studied medicinal plants, *F mollis* and *A. fragrantissima*, mainly belong to *Bacillus* and *Brevibacillus* spp. Bacterial endophytes characterization including extracellular enzymatic activity, antimicrobial actions, P-solubilization activity, ammonia, and IAA production were performed in terms of their plant growth-promoting abilities in-vitro and in plants. Five bacterial strains identified as *Brevibacillus agri* Af.13, and Af.14, *Bacillus* sp. Fm3, *Bacillus* sp. Fm.4, and *Bacillus* sp. Fm.6 were selected and inoculated into maize plant to increase their growth performance under normal conditions. These endophytic bacterial isolates significantly promote plant growth and increase P and N shoot contents of maize plant. However, in order to demonstrate the beneficial role of these bacterial endophytes in plant growth promotion of their host plants, particularly under real field conditions, further investigation of their mechanisms of colonization and competition against other soil microbial communities will be required.

Author Contributions: Conceptualization, A.F., A.M.E., E.E.-D.E., S.E.-D.H., K.A.A.A., Y.M.H., K.A.A. and M.D.F.A.; methodology, A.F., A.M.E., E.E.-D.E., S.E.-D.H., K.A.A.A., Y.M.H., K.A.A. and M.D.F.A.; software, A.F., A.M.E., F.A.-O., M.H., M.S.-A., E.E.-D.E., S.E.-D.H., K.A.A.A., Y.M.H., K.A.A. and M.D.F.A.; formal analysis, A.F., A.M.E., E.E.-D.E., S.E.-D.H., F.A.-O., M.H., M.S.-A., K.A.A.A., Y.M.H., K.A.A., A.M.E. and N.K.; investigation, A.F., A.M.E., E.E.-D.E., S.E.-D.H., F.A.-O., M.H., M.S.-A., K.A.A.A., and N.K.; resources, A.F., A.M.E., E.E.-D.E., S.E.-D.H., K.A.A.A., Y.M.H.; data curation, A.F., A.M.E., E.E.-D.E., S.E.-D.H., F.A.-O., M.H., M.S.-A., K.A.A.A., Y.M.H. and K.A.A.; writing—original draft preparation, A.F., A.M.E., E.E.-D.E., F.A.-O., M.H., M.S.-A., S.E.-D.H. and K.A.A.A.; writing—review and editing, A.F., A.M.E., E.E.-D.E., S.E.-D.H., K.A.A.A., Y.M.H., K.A.A., A.M.E., M.A. and N.K.; visualization, A.F., A.M.E., E.E.-D.E., S.E.-D.H., K.A.A.A., Y.M.H. and K.A.A.; funding acquisition, M.D.F.A., K.A.A., Y.M. and K.A.A.A. All authors have equal contribution. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Deanship of Scientific Research at Princess Nourah bint Abdulrahman University through the Fast-track Research Funding Program.

Acknowledgments: The authors extend their appreciation to the Deanship of Scientific Research at Princess Nourah bint Abdulrahman University for funding this research through the Fast-track Research Funding Program.

Conflicts of Interest: The authors declare no conflict of interest.

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