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Plant Growth-Promoting Bacterium from Non-Agricultural Soil Improves Okra Plant Growth

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Abstract: Beneficial soil microorganisms influence nutrient recycling, soil fertility, plant growth, and productivity and reduce chemical fertilizer application. This study aimed to isolate bacteria from non-agricultural soils in the Al-Ahsa region and characterize the bacteria with the best biostimulating characteristics at the physiological, biochemical, and molecular level. DPM17, a bacterial isolate, promotes plant growth through phosphate solubilization, nitrogen fixation, and ammonia production. DPM17 also produces the phytohormones, indole acetic acid (IAA; 4.516 μg mL^{-1}) and gibberellin (1.33 μg mL^{-1}), and ammonia (0.06 μg mL^{-1}). Additionally, DPM17 grows in the presence of up to 10% NaCl, indicating its halophilic nature. DPM17 was identified as *Bacillus baekryungensis* based on comparative sequence analysis of the 16S rRNA gene, and neighbor-joining phylogenetic analyses indicated that DPM17 was 96.51% identified to *Bacillus* sp. DPM17 inoculation substantially improved *Abelmoschus esculentus* (okra) root length, lateral root count, and dry weight from 7.03 to 9.41 ($p = 0.03$), 3.2 to 7.2, and 6 to 13 mg ($p = 0.032$), respectively. The results suggest that DPM17 enhances plant growth and can be exploited to develop efficient formulations for sustainable agriculture and food security in Saudi Arabia.

Keywords: *Bacillus*; *Abelmoschus esculentus*; biostimulation; non-agricultural soils

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1. Introduction

Soil microbiota function in nutrient recycling, such as carbon and nitrogen, enhancing soil quality, increasing crop yield, and decreasing the application of chemical fertilizers [1]. Plant growth-promoting bacteria (PGPB) stimulate plant growth via bioactive compound production, iron sequestration, and inorganic phosphate solubilization, among others. PGPB also enhance the rhizoremediation of petroleum hydrocarbons and phenol biodegradation [2,3] and reduce heavy metal uptake by plants [4]. Thus, they can be used for a wide range of industrial and biotechnological applications.

Abelmoschus esculentus (okra) is important in the human diet due to its high carbohydrate, fat, protein, mineral, and vitamin content. It is also used in medicine and industry [5]. The low bioavailability of essential nutrients and high salinity of soils negatively affect okra growth and productivity in Saudi Arabia [6]. Increasing okra production via applications of synthetic chemical fertilizers has potential hazards to the environment and associated organisms. Hence, searching for a safe, eco-friendly, and efficient strategy to increase okra production is of high significance.

Various microbial groups, including *Aeromonas encheleia* and *Pseudomonas azotoformans* isolated from earthworms, are affiliated with PGPB [7]. The biostimulation properties of PGPB isolated from Al-Ahsa, Saudi Arabia, have been investigated. *Bacillus megaterium* and *Enterobacter cloacae* were isolated from *Medicago sativa* root nodules and roots,

respectively [8–11]. *Pseudomonas monteilii* was isolated from *Solanum lycopersicum* [12]. However, to the best of our knowledge, no previous studies have focused on PGPB isolated from non-agricultural soils in Al-Ahsa. Additionally, PGPB from such non-agricultural soils are more likely to have properties not present in those from agricultural soils. This could be due to harsh conditions, such as salinity, drought, and a lack of adequate nutrients in non-agricultural soils. Salinity is a serious global issue that has a deleterious effect on global food security. Additionally, halophilic/halotolerant PGPB have a particular advantage over halophobic PGPB, allowing them to survive and increase crop yield in saline environments. Hence, this study aimed to explore PGPB in such soils. The specific aims were to obtain and estimate the enhancing properties of the bacterial isolate (PGPB) from non-agricultural soils on okra plants in Al-Ahsa, Saudi Arabia and, then, identify and characterize the best isolate.

2. Materials and Methods

2.1. DPM17 Isolation and Parameters of the Soil Sample

One 5 cm deep surface soil sample was collected in a sterile container from a non-agricultural area in Al-Ahsa, Saudi Arabia (25°37′35.10″N, 49°59′94.19″E) on 15 March 2018. Then, 1 g of the dry soil was added to 9 mL of a saline solution (0.85% NaCl), mixed thoroughly for 5 min, and serially diluted. A 0.1 mL aliquot was spread over a nutrient agar (NA) plate and incubated at 28 °C for 48 h. Single colonies were then purified on fresh agar plates using the streaking method. The bacterial isolates were cryopreserved in glycerol (20% *v*/*v*) and stored at −20 °C.

The salinity and pH of the soil samples were determined using a pH meter according to a previously described method [13]. Briefly, 5 mL of distilled water was added to 5 g of dried soil, which were mixed together, filtered, and allowed to stand for 30 min. Then, the pH was measured by immersing the pH meter electrode in the soil solution after calibration. The salinity was measured in the supernatant of the soil solution using a pH meter in salinity measurement mode.

2.2. Phenotypic Characterization

DPM17 was characterized based on colonial features, including shape, color, margin, and elevation, and the Gram staining reaction was determined for the cells. Moreover, its growth on 20 different metabolites containing API-20E (bioMérieux, Marcy l'Etoile, France) was checked by incubating for 24–48 h at 30 °C following the manufacturer's instructions. DPM17 growth under salinity stress was also determined by growing on 0, 3, 5, 7, or 10% NaCl-supplemented NA plates at 30 °C for 48 h. The experiment was repeated thrice [14]. The starch-hydrolyzing ability of DPM17 was tested by inoculating it on 10% starch-supplemented NA plates. After 24–48 h of incubation at 30 °C, the formation of a clear zone around the colonies after adding iodine solution was examined [15]. Catalase production by DPM17 was tested by adding a drop of 5% H_2O_2 to an aliquot of an actively growing strain. The formation of gas bubbles was considered positive for catalase enzyme [16].

2.3. Genotypic Characterization Using 16S rRNA Gene Sequence

DNA was extracted from mid-exponential phase DPM17 cells by boiling an aliquot at 95 °C for 5 min and then centrifuging at 10,000 rpm for 10 min to precipitate the cells [17]. Briefly, 1 µL supernatant was used as the template for subsequent PCR amplification of the 16S rRNA gene using the primer pair 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R 5′-TACGGYTACCTTGTTACGACTT-3′. The PCR conditions for 30 amplification cycles have been described previously [10]. The PCR amplicons were then analyzed by electrophoresis on a 1% agarose gel in a 100 mL 1× TAE buffer. The amplicon size was determined using a 1 kb DNA ladder. The 16S rRNA gene of DPM17 was sequenced using an automated DNA sequencing system (Applied Biosystems model 3730XL, Foster City, CA, USA). Phylogenetic trees were constructed using the freely available software MEGA7 (version 7.0, Molecular Evolutionary Genetics, Philadelphia, PA, USA) [18]. The evolutionary history was inferred using the neighbor-joining method [19]. The optimal tree

with a sum of branch length = 0.38845289 is shown. The evolutionary distances were computed using the maximum composite likelihood method involving eight nucleotide sequences including first, second, third, and noncoding nucleotides and are expressed as the number of base substitutions per site [20]. All positions containing gaps and missing data were eliminated. The final dataset contained 1343 positions. Evolutionary analyses were conducted using MEGA7 [18].

2.4. Plant Growth Promoting Features

The nitrogen-fixing capability of DPM17 was assessed by incubating it on nitrogen-free Jensen's agar medium for 72 h at 30 °C. Positive results were scored based on the appearance of DPM17 colonies [21].

Phosphate solubilization assays were performed by streaking DPM17 on Pikosvkaya agar containing calcium triphosphate ($Ca_3(PO_4)_2$) as the phosphate source (5 g), yeast extract (0.50 g), dextrose (10 g), ammonium nitrate (NH_4NO_3, 0.50 g), sodium chloride (NaCl, 0.20 g), magnesium sulfate ($MgSO_4$, 0.10 g), and agar (15 g) [22]. After incubating at 30 °C for 72 h, clear halo zone formation around DPM17 colonies was recorded as a positive result [22].

Indole acetic acid (IAA) production by DPM17 was examined using a previously described method [23]. Briefly, DPM17 cells were grown in a nutrient broth supplemented with tryptophan (0.2 mg mL^{-1}) and incubated in a shaking incubator for 72 h at 30 °C. The cells were precipitated by centrifugation at 10,000 rpm for 10 min. Next, 1 mL of the supernatant was mixed with 2 mL of Salkowski reagent (150 mL concentrated H_2SO_4, 250 mL distilled water, and 7.7 mL 0.5 M $FeCl_3 \cdot 6H_2O$) and incubated in dark at 30 °C for 25 min. The amount of IAA produced was estimated from the absorbance at 530 nm, measured using a spectrophotometer ($OD_{530\,nm}$).

The ammonia production by DPM17 was tested by incubating with peptone water for 72 h at 30 °C. The supernatant was separated from cells by centrifugation at 10,000 rpm for 15 min. Next, 1 mL of the supernatant was mixed with 1 mL of Nessler's reagent and 8 mL of distilled water. The formation of a yellow color indicated ammonia production. Measurements were performed in triplicate. The amount of ammonia was estimated from the absorbance at 450 nm, measured using a spectrophotometer ($OD_{450\,nm}$) and a standard curve of 0.1–1 µmol mL^{-1} ammonium sulfate [24].

The ability of DPM17 to produce gibberellin was tested by growing it in a nutrient broth (NB) medium at 30 °C for 72 h. The cells were precipitated by centrifugation at 8000 rpm for 10 min. Then, 2 mL of a zinc acetate solution was added to 15 mL of the supernatant, mixed thoroughly, and incubated for 2 min. After adding 2 mL of potassium ferrocyanide, the tubes were centrifuged at 8000 rpm for 10 min. Next, 5 mL of the mixture was gently mixed with an equal volume of 30% HCl and incubated at 30 °C for 75 min. The amount of gibberellin was estimated from the absorbance at 254 nm, measured using a spectrophotometer ($OD_{254\,nm}$) and a 0.1–1 µmol mL^{-1} gibberellin standard curve [25].

2.5. Inoculation of Okra Plants with DPM17

This in vitro experiment was conducted to assess the DPM17-mediated enhanced growth of okra following a previously described method [8]. Healthy okra seeds were immersed in 10 mL of 70% ethanol for 10 s, then in 10 mL of 3% H_2O_2 for 1 min, and subsequently rinsed several times with sterile distilled water. The surface-sterilized seeds were planted in pots (five seeds per pot) containing agricultural soils that had been sterilized three times. Mid-exponential phase DPM17 (5 mL $OD_{660\,nm}$: 1.0, ~3×10^8 CFU.mL^{-1}) was immediately added to the seeds. Untreated control plants received an equal volume of water. The treatments were repeated three times. The growth chamber conditions were 25 °C and a 14/10 h light/dark cycle. After two weeks, the okra plants were uprooted, and the dry weight, root and shoot heights, and the lateral root count of treated and untreated plants were reported.

2.6. Statistical Analysis

The pot trial was conducted as a factorial experiment in a completely randomized design. Statistical analyses of data were performed using analysis of variance (ANOVA), and the mean comparisons were performed by post hoc tests using Microsoft Excel. All assays were performed in triplicate (n = 15), and the values were expressed as mean ± standard deviation (SD). Significant differences were scored at p-value ≤ 0.05.

3. Results

3.1. Soil Parameters and Isolation and Characterization of the PGPB

The pH of the soil sample was 7.35, highlighting slight alkalinity. The salinity level of the soil was 10 dS m^{-1}, indicating that it was saline. DPM17, a bacterial isolate recovered from a non-agricultural area in Al-Ahsa, Saudi Arabia, exhibited plant growth-promoting traits.

The morphological, biochemical, plant growth-promoting, and genotypic features of DPM17 are presented in Table 1. DPM17 developed white circular colonies with undulate margins and raised colony surfaces on NA for 72 h at 30 °C. The DPM17 cells were rod-shaped and reacted positively to Gram staining (Table 1).

Table 1. Polyphasic DPM17 characterization.

Morphological Features	DPM17
Colony shape	Circular
Color	White
Colony diameter	1–3 mm
Colony elevation	Raised
Colony margin	Undulate
Gram reaction	gram-positive
ONPG * (Beta-galactosidase)	+
Arginine (Arginine dihydrolase)	+
Lysine (Lysine decarboxylase)	−
Ornithine (Ornithine decarboxylase)	−
Citrate (Citrate utilization)	−
Na thiosulfate (H_2S production)	−
Urea (Urea hydrolysis)	−
Tryptophan (Deaminase)	+
Indole (Indole production)	+
Na pyruvate (Acetoin production)	+
Charcoal gelatin (Gelatinase)	+
Glucose (Fermentation/oxidation)	+
Mannitol (Fermentation/oxidation)	+
Inositol (Fermentation/oxidation)	+
Sorbitol (Fermentation/oxidation)	+
Rhamnose (Fermentation/oxidation)	−
Sucrose (Fermentation/oxidation)	+
Melibiose (Fermentation/oxidation)	+
Amygdalin (Fermentation/oxidation)	+
Arabinose (Fermentation/oxidation)	+
Catalase (Oxidation/reduction)	+
Amylase (Starch hydrolysis)	+
Growth under salt stress (0–10% NaCl)	+
Nitrogen fixation	−
Phosphate solubilization	+
Indole acetic acid (IAA) production	4.51–5.04 μg mL^{-1}
Ammonia production	0.06 μg mL^{-1}
Gibberellin (GA3) production	1.33 μg mL^{-1}
Catalase test	+
Closest strain	*Bacillus* sp.
Identity (%)	96.51
Accession number	MT949889

*: ortho-Nitrophenyl-β-galactoside, (+) indicates positive results and (−) indicates negative results.

API-20E is a commercial kit used for rapid biochemical characterization and identification and determines 20 biochemical traits. DPM17 consumed 65% (13 of 20) of the biochemical substrates in the API-20E microtests. Unlike rhamnose, DPM17 metabolized the assayed carbohydrates (Table 1). However, among all the amino acids tested, DPM17 consumed only arginine. Similarly, the strain did not grow on citrate, sodium thiosulfate, or urea, demonstrating a lack of the respective metabolic enzymes. Remarkably, DPM17 produced acetoin, an elicitor that may induce plant resistance against phytopathogens. A clear halo zone developed around the DPM17 colonies upon adding iodine drops, demonstrating amylolytic activity (Table 1). DPM17 exhibited good growth under 0–10% NaCl, indicating its halophilic nature (Figure 1).

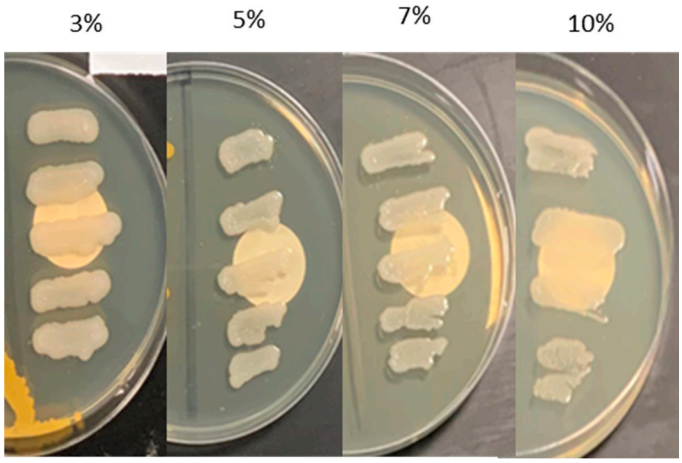

Figure 1. DPM17 growth under different NaCl concentrations.

BLAST analysis of the 16S rRNA gene showed that DPM17 was affiliated with *Bacillus* sp. PK5-4 (accession number AY505506.1) with 96.51% identity (Table 1). Phylogenetic analysis showed that DPM17 clustered with *Bacillus baekryungensis* PK5-4 (accession number: AY505506), indicating that they have the same monophyletic origin (Figure 2).

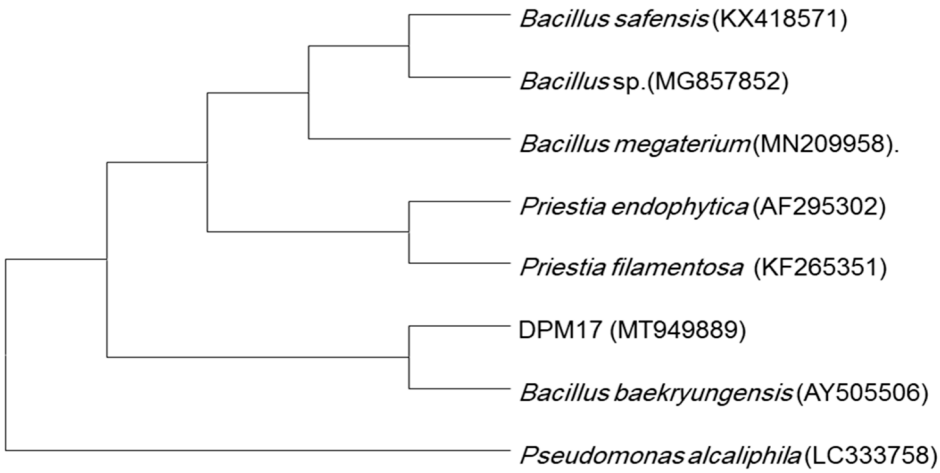

Figure 2. The evolutionary relationships of DPM17 and closely related bacterial species.

DPM17 grew well on Jensen's medium containing no added nitrogen, suggesting its nitrogen fixation capability (Table 1 and Figure 3). A clear zone was formed around DPM17 colonies on plates containing Pikovskayas agar medium, indicating that it solubilizes phosphate (Table 1). DPM17 produced IAA (4.516 μg mL^{-1}) in the absence of tryptophan. When 0.30 μg mL^{-1} tryptophan was added, DPM17 produced 5.04 μg mL^{-1} IAA (Table 1). DPM17 also produced ammonia (0.06 μg mL^{-1}), as indicated by the formation of a yellow

color upon adding the Nessler's reagent, and 1.33 μg mL^{-1} gibberellin (Table 1). DPM17 was catalase positive, as evidenced by immediate gas bubble development after gently mixing H_2O_2 with the actively grown cells (Table 1).

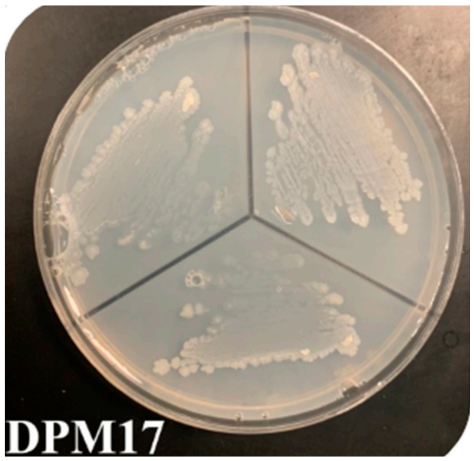

Figure 3. DPM17 growth on Jensen's N-free medium.

3.2. Effect of DPM17 on Okra Growth

DPM17 substantially enhanced the growth parameters of okra (Table 2 and Figure 4). DPM17 inoculation significantly promoted root length and lateral root count from 7.03 and 3.2 to 9.41 and 7.2, respectively (p = 0.03 and 0.04, respectively; Table 2), remarkably enhancing the okra root system. Additionally, the dry weight of DPM17-inoculated okra roots increased from 6.34 mg to 13.3 mg (p = 0.032; Table 2). The stem length or dry weight changed significantly after inoculation with DPM17 (Table 2).

Table 2. Effects of DPM17 inoculation on the growth parameters of okra plants.

Growth Parameter	Control	DMP17-Treated
Root length (cm)	7.03 ± 0.33	9.41 * ± 0.34
Stem length (cm)	17.86 ± 0.27	19.1 * ± 1.16
Lateral root count $plant^{-1}$	3.2 ± 0.44	7.2 * ± 0.83
Dry weight root $plant^{-1}$ (mg)	6.34 ± 0.23	13.3 * ± 0.25
Dry weight $shoot\ plant^{-1}$ (mg)	26.1 ± 0.41	28.3 * ± 0.47

* Statistically significant difference ($p < 0.05$).

Figure 4. Effect of DPM17 on okra plant growth.

4. Discussion

This study aimed to explore the PGPB from non-agricultural areas of Al-Ahsa city, Saudi Arabia, and assess their ability to stimulate growth parameters in okra. DPM17, a bacterial isolate, was selected for phenotypic, biochemical, and biostimulation characterization, and genotypic identification based on 16SrRNA gene sequencing.

The microbial composition, diversity, and abundance in soil are remarkably affected by various agricultural practices and chemical and physical factors, including soil salinity and pH. The pH of the non-agricultural soil was 7.35, demonstrating the slight alkalinity of the Al-Ahsa soils. This finding is consistent with that previously reported by Suleiman and Sallam (2016) for Al-Ahsa soils [26]. The soil sample displayed relatively high electrical conductivity (10 dS m^{-1}), demonstrating the slight salinity of the soil under consideration. Various reasons could explain soil salinity, including poor rainfall that deposits salt on the soil surface [27]. Moreover, Nawar et al. (2014) have also reported the salinity in the soils of Al-Ahsa (8–16 dS m^{-1}) [28]. Based on the United States Department of Agriculture (USDA) classification of soils, non-agricultural soil is classified as moderately saline soil. The electrical conductivity (EC) of a given soil is an estimation of the soil salinity, that is, the overall salts dissolved in the soil. Soil salinity affects nutrient absorption from the soil, thereby affecting soil fertility and the availability of plant nutrients.

Elevated salinity is a major environmental concern and negatively impacts food security worldwide [29]. Good DPM17 growth under high NaCl concentration coincides with that reported for *Bacillus* sp., which could grow at 12% NaCl [10,30,31]. According to the classification of halophilic organisms, DPM17 is a moderately halophilic PGPB, as evidenced by growth at 10% NaCl. Additionally, halotolerant PGPB inoculation promotes the yield of many crops in saline soils [32]. Halophilic/halotolerant PGPB possess a selective advantage over halophobic PGPB, enabling them to exist in saline localities [9].

DPM17 displayed metabolic diversity, as evidenced by the results obtained using API-20E strips. This observation is in general agreement with those reported for *Bacillus* spp. strains [33,34]. This indicates the versatility of the metabolic pathways of DPM17 and its significance in improving plant growth and yield via multiple mechanisms, including ammonia production with the help of the arginine dihydrolase enzyme or indirectly via acetoin production. Two enzymes, α-acetolactate decarboxylase and acetolactate synthase, are involved in acetoin production, which plays an important role in inducing plant systemic resistance against plant microbial pathogens.

Further evidence of the biostimulation capabilities of DPM17 was its ability to fix nitrogen, solubilize phosphate, and produce IAA, gibberellin, and catalase. DPM17 cells were grown on nitrogen-free Jensen agar. Nitrogen fixation is mediated by a multienzyme complex called the nitrogenase enzyme. This may indicate that DPM17 is a nitrogen-fixing bacterium and possesses a nitrogenase enzyme. This observation is in general agreement with those reported in other studies [11,34]. Nitrogen-fixing PGPB can significantly satisfy nitrogen requirements for plants, thus improving soil health and crop yield.

The ability of DPM17 to produce growth regulators (IAA and gibberellins) also confirms its plant-stimulation capability. IAA is a plant hormone that improves seed germination and the growth of different plant organs even at 10^{-9}–10^{-12} M [35,36]. In this study, DPM17 produced 5.04 μg mL^{-1} IAA, which is similar to that produced by *Bacillus* sp. [37]. However, substantially higher IAA production (128.5 μg mL^{-1}) than our findings have been recently reported [35], indicating variability in IAA production by bacterial strains. Many factors affect IAA production, including culture growth and estimation methods. These observations show that IAA production is a key trait of PGPB, including DPM17.

Gibberellins are growth phytoregulators that regulate seed germination, root and stem growth, and leaf and fruit senescence [38]. DPM17, as a PGPB, produced 1.37 μg mL^{-1} gibberellin, which is apparently higher than that (0.002–015 μg mL^{-1}) reported previously [39,40].

DPM17 produced ammonia, which is consistent with the ammonia production by *Bacillus* strains [41]. DPM17 produced less ammonia (0.06 μg mL^{-1}) than other *Bacillus* strains (0.16–0.96 μg mL^{-1}) obtained from plum trees (*Prunus domestica*), indicating that the

quantities of ammonia produced differ among bacterial strains. Plants and microbes use ammonia for amino acid synthesis and breakdown through various enzymatic pathways. Furthermore, ammonia acts as an anti-phytophogenic agent [41]. It can be assumed that the ammonia concentration determines its function. When less ammonia is produced, it is likely to act as a nitrogen source for plants. However, when more ammonia is produced, it can exhibit antimicrobial activity against plant pathogens. *Pseudomonas stutzeri* and *B. toyonensi*, which produce high levels of ammonia, exhibit significant antifungal activities against *Verticillium dalhiae* and *Fusarium oxysporum*, which cause wilt diseases in *Solanum lycopersicum* (tomato) and *Cucumis melo* (melon), respectively. Consequently, DPM17, as an ammonia-producing PGPB, improves the health of plants and their yield directly or indirectly.

DPM17 possesses a catalase enzyme that has a protective role against free radical toxicity. This PGPB trait of DPM17 is in accordance with that of *Bacillus* sp. [10,34]. Environmental stressors cause the generation of toxic free radicals, which attack vital macromolecules within organisms. Thus, catalase-producing PGPB, including DPM17, indirectly mediate plant biostimulation.

The results from the comparative analysis of 16S RNA sequencing showed that DPM17 seem to belong to *B. baekryungensis* with 96.51% sequence identity. Additionally, DPM17 clustered with the *Bacillus* clade based on the generated neighbor-joining phylogenetic tree. Our phylogenetic results are consistent with those of the taxonomic and morphological characterization of DPM17, providing robustness to the affiliation of the bacterial strain. Additionally, colonial characteristics and the Gram staining reaction of DPM17 were in general agreement with those reported for *Bacillus* sp. [42].

The multiple biostimulating activities of DPM17 were verified by their beneficial effects on the growth parameters of okra plants. Root length, dry weight, and branching was significantly enhanced in okra inoculated with DPM17. These observations are consistent with the comparable positive effects of *Bacillus* spp. in various plant species, including *Pisum sativum*, *Lens esculentus*, *Phaseolus vulgaris*, *Triticum vulgare*, and *Solanum lycopersicum* plants [8,10,34,43]. The improvement in the okra root growth parameters illustrated herein could be explained by the multiple biostimulation features of DPM17.

5. Conclusions

In conclusion, this study reported the isolation of DPM17, a halotolerant bacterial strain, from a non-agricultural area in the Al-Ahsa region. *Bacillus baekryungensis* exhibited multiple growth-enhancing features, such as ammonia, IAA, and gibberellin (GA3) production; phosphate solubilization; and nitrogen fixation; therefore, it improved the growth parameters of *Abelmoschus esculentus* (okra plant). Thus, DPM17 can be exploited for developing green biofertilizers for sustainable agriculture and food security.

Author Contributions: H.A.A.: methodology, data curation. A.K.; conceptualization, writing—original draft preparation, writing—reviewing and editing, supervision, project administration and funding acquisition. M.A.; writing—reviewing and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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