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IDENTIFICATION AND CHARACTERIZATION OF PLANT-GROWTH-PROMOTING BACTERIAL ENDOPHYTES FROM DIFFERENT PARTS OF *PHOENIX DACTYLIFERA*

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Abstract

The rapid population growth and climate change underscore the need for a sustainable food supply. Plant growth-promoting (PGP) bacteria present an eco-friendly and cost-effective solution by enhancing plant growth, improving stress resilience, and reducing reliance on chemical inputs. This study aimed to isolate and characterize bacterial endophytes from *Phoenix dactylifera* (date palm) and evaluate their potential as biofertilizers and biocontrol agents. A total of 56 bacterial strains were isolated from *Phoenix dactylifera* (date palm) to evaluate their plant growth-promoting properties. Growth conditions were optimized, revealing that most isolates thrived at pH 7, with reduced growth at temperatures above 30°C. Salt stress also affected growth, with densities dropping by 31% at 3% salt and 53% at 5% salt compared to 1% salt levels. All bacterial isolates demonstrated strong plant growth-promoting traits: IAA from 0.60 to 10.13 µg/ml, 93% generated ammonia, 82% fixed nitrogen, and 41% solubilized phosphate. Citrate production was observed in 54% of isolates, while 87% and 20% solubilized zinc and potassium, respectively. Amylase and protease activities were found in 70% and 79% of isolates, and 96% exhibited catalase activity. Out of 56 bacterial isolates, 15 top-performing strains were further analyzed. Those isolates exhibited cellulase, asparaginase activity, and hydrogen cyanide production. Additionally, 93% produced lipase, 60% had pectinase activity, and 13% produced esterase. The selected 15 strains exhibited resistance to zinc, nickel, and lead. Additionally, 87% were resistant to copper, and 13% to cadmium. Those strains were resistant to streptomycin, and 20% were resistant to kanamycin. Those isolates also displayed antifungal activity against *Alternaria brassicicola*, and 27% were effective against *Aspergillus niger*. Molecular identification via 16S rRNA gene sequencing revealed the isolates as *Bacillus safensis, B. paranthracis, B. paramycoides, B. tropicus, B. aerius, B. anthracis, Peribacillus acanthi and Haemophilus parainfluenzae*. The findings highlight the potential of *Phoenix dactylifera* (date palm) derived bacterial endophytes as multifunctional biofertilizers and biocontrol agents. Their robust PGP traits and resistance to environmental stresses suggest promising applications in sustainable agriculture, contributing to improved crop productivity and resilience in the face of global challenges.

Key words: Endophytic bacteria, *Phoenix dactylifera*, Biofertilizer, Biocontrol properties, Molecular identification.

Abbreviations: Carboxy-Methyl-Cellulose (CMC); CTAB: Cetyltrimethyl ammonium Bromide; DNA: Deoxyribonucleic Acid; Hydrogen Cyanide (HCN); IAA: Indole-3-acetic acid; LB: Luria–Bertani; NCBI: National Center for Biotechnology Information; PCR: Polymerase Chain Reaction; PDA: Potato Dextrose Agar; PGP: Growth-Promoting Properties; PGPB: Plant Growth Promoting Bacteria; PL: *Phoenix dactylifera* Leave; PR: *Phoenix dactylifera* Root; PS: *Phoenix dactylifera* Stem.

Introduction

The continuous increase in human population and the effects of climate change have elevated the significance of addressing food security as an urgent and critical challenge. At present, the global population stands at approximately 8 billion individuals and worldwide agricultural productivity needs to be increased by 1.73% on an annual basis to cater to the increasing food demand of 10 billion people by 2050 (Anon., 2020). Chemical fertilizers, particularly those containing inorganic nitrogen, are heavily used by farmers to meet the food demands of this rapidly increasing population. In typical inorganic fertilizer applications, 60-90% are not beneficially used by the crops, and the remaining 10-40% are used by the plants. The uncontrolled application of chemical fertilizers poses a significant danger to the environment, leading to pollution of the air, water, and soil. The use of these chemicals has adverse effects on soil fertility, including reduced water retention capacity, increased salinity, imbalanced soil nutrients, and compromised fertility (Savic, 2012).

For maximum benefits, both in terms of fertilizer savings and improved plant growth, it is crucial to apply plant-growth bacteria at the appropriate time during the fertilization process. This strategic timing ensures maximum benefits in terms of improved plant growth and efficient utilization of fertilizers (Hungria *et al.*, 2010; Adesemoye *et al.*, 2009). Bio-fertilizers offer several advantages over chemical fertilizers because they are affordable, renewable, and do not pollute the environment. Biofertilizers fix biological nitrogen and solubilize or mineralize phosphate and potassium (Sinha *et al.*, 2014; Singh *et al.*, 2011).

Recently, numerous Plant Growth Promoting Bacteria (PGPB) have been introduced into the market as biofertilizers or biocontrol agents (Calvo *et al.*, 2014; Reed & Glick, 2013). The date palm (*Phoenix dactylifera*), a monocotyledonous and dioecious plant of the *Arecaceae* family, is cultivated in arid and semiarid regions of North Africa, and the Middle East and arid and semi-arid regions of Pakistan, it can tolerate drought, soil fertility, and salinity(Ait-El-Mokhtar *et al.*, 2020; Chao & Krueger, 2007). Therefore in this study, bacterial strains were

isolated from different parts of *Phoenix dactylifera* (date palm) with plant growth-promoting properties, using morphological, biochemical, and molecular methods, to evaluate their potential as bio-inoculants (bio-fertilizers).

Material and Methods

The experiment included isolating, identifying, and characterizing endophytic bacteria from *Phoenix dactylifera L.* and the study was conducted at the Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture Peshawar, Pakistan.

Collection of plant samples and surface sterilization: For isolation of endophytes, healthy leaf, stem, and root samples were collected from location (*Phoenix dactylifera* 34.021075N 71.467607E) of district Peshawar, Khyber Pakhtunkhwa, Pakistan, and brought to the laboratory immediately for processing. The surface sterilization procedure was carried out with some modifications based on the method described by Li *et al.*, 2017. The modified surface sterilization procedure involved washing the samples to remove visible particles, disinfecting them with 75% ethanol (3 minutes) and 20% sodium hypochlorite (3 minutes), washing with Tween-20 (1 minute), and rinsing with autoclaved distilled water under controlled conditions.

Endophytic bacteria's isolation and growth: Both methods isolated endophytic bacteria from different plant tissues (leaves, roots, and stems). The first method involves cutting the tissue into small pieces and placing them on LB agar plates (Anjum & Chandra, 2015), while the second method involved macerating the tissue and spreading the resulting supernatant on LB agar plates (Li *et al.*, 2017). Morphologically distinct colonies were selected, streaked, and purified before being stored for further study.

Identification of endophytic microorganisms through molecular technique

Extraction of bacterial genome: The CTAB DNA extraction method isolated total DNA from a bacterial culture. The extracted DNA was then quantified using a NanoDrop spectrophotometer, providing information on the DNA concentration and quality for further analysis or experimentation.

16SrRNA gene amplification: The PCR amplification of the 16S rRNA gene from the genomic DNA was obtained using the CTAB DNA extraction method. Universal primers, specifically 27F, and 1492R, were used for the amplification(Chelius and Triplett, 2001).

27F 5′AGAGTTTGATC (AC) TGGCTCAG-3′
1492R 5′-CGG (CT) TACCTTGTTACGACTT-3′

The reaction mixture contained a 5 µl Green Master Mix, 0.2 µl Taq polymerase, 0.5 µl forward and reverse primers, 1 µl template DNA, and 2.8 µl PCR water to reach the final volume of 10 µl. Amplification was performed in a thermocycler program as follows: pre-denaturation (95°C, 3 minutes), followed by denaturation (95°C, 30 sec), annealing (55°C, 30 seconds), elongation (72°C, 1 minute), post elongation (72°C for 10 minutes). The denaturation phase, primer attachment, and elongation processes were carried out for 34 cycles. The resulting PCR products can be used for further analysis, such as sequencing or characterization of the bacterial strains. After the PCR amplification of the 16S rRNA gene, the resulting PCR product was visualized on a 1% agarose gel containing ethidium bromide. The DNA bands were visualized using a gel documentation system.

Sequencing and phylogenetic analysis of bacterial isolates: For Sanger sequences of 16SRNA, the purified PCR product was sent to Macrogen (South Korea) and BGI, Hong Kong. The quality of the sequences was assessed, and trimming was performed using BioEdit software. The percentage similarity index for the sequenced strains was assessed using BLAST-NCBI software, and the resulting sequences were submitted to NCBI-GenBank to obtain accession numbers.

Morphological studies: The bacterial isolated colonies were morphologically classified based on the gram reaction, size, shape, color, margin, and elevation.

Growth characteristics of endophytic bacteria under different conditions: The bacterial isolates were tested for growth in temperatures (15°C, 28°C, and 40°C) over 5-7 days. The pH range was tested by adjusting a liquid LB medium containing cultures to different pH values ranging from 4 to 10 (Fterich *et al.*, 2012). The bacteria were grown in a liquid LB medium with NaCl concentrations of 0%, 1%, 3%, and 5% at 30°C for 24 hours in a shaking incubator. After 24 hours, their growth was measured by taking absorbance at 600 nm using a blank, non-inoculated broth (Li *et al.*, 2017).

Screening of endophytic bacteria for growth-promoting traits

Indole acetic acid production: The endophytic bacteria were grown in 5 ml of LB broth with and without L-tryptophan and incubated at 30°C and 200 rpm for one day. The bacterial inoculum was then centrifuged at 10,000 rpm for 10 minutes, and the resulting supernatants were transferred to new tubes. One part of the supernatant was mixed with two parts of Salkowski's reagent and kept in the dark for half an hour. The resulting pink color was measured at 535 nm using a spectrophotometer. This procedure was repeated three times, and the mean value was recorded. The concentration of IAA was estimated for each sample using a standard IAA curve (Gordon & Weber, 1951).

Phosphate solubilization: The ability of the bacterial isolates to solubilize Phosphate was examined based on the formation of a clear halo zone on Pikovskaya agar medium incubated for 3 days at 28°C (g L^{-1}: dextrose-10.0; yeast extract-0.5; ammonium sulfate-0.5; calcium phosphate-5.0; potassium chloride-0.2; magnesium sulfate-0.1; ferrous sulfate-0.001; manganese sulphate-0.001; and agar-15.0) (Pikovskaya, 1948). The isolates forming a clear halo zone around the colonies were considered positive.

Nitrogen fixation: The endophytic bacteria's ability to fix nitrogen was evaluated by their growth in Jensen media (Jensen, 1942). The bacterial isolates' colonies were streaked on Jensen's medium and Bromothymol Blue for 4 to 8 days at 28°C to observe growth (gL^{-1}: dipotassium phosphate-1.0; sucrose-20.0; magnesium sulfate-0.5; sodium chloride-0.5; sodium molybdate-0.005; ferrous sulfate-0.1; calcium carbonate-2.0; agar-15.0). A color change from green-blue to dark blue indicated a positive result.

Zinc solubilization: The bacterial isolates were tested for their ability to solubilize zinc using a minimal Tris medium supplemented with zinc oxide at a concentration of 0.1% Zn and adjusted to pH 7 (gL^{-1}: D-glucose-10; KCl-1.49; NaCl-4.68; Tris-HCl-6.06; NH_4Cl-1.07; Na_2SO_4-0.43; $MgCl_2.2H_2O$-0.2; $CaCl_2.2H_2O$-0.03; and agar-15). Spot inoculation of the bacterial isolates was carried out, followed by incubation for 7 days in the dark at 28°C. The isolates were then examined for a clear halo zone around bacterial growth, indicating the solubilization of zinc (Fasim *et al.*, 2002).

Potassium solubilization: The ability of the bacterial isolates to solubilize Potassium was examined on modified Aleksandrov agar medium plates (gL^{-1}: glucose-5; calcium carbonate-1; magnesium sulfate heptahydrate-0.5; iron chloride-0.005; calcium phosphate-2; feldspar/ mica-2 and agar-18 at pH-7.2). The plates were inoculated with spots of bacterial isolates and then incubated at 30°C for 24-72 hours to observe the development of a clear zone. The appearance of this clear zone indicated the solubilization of Potassium (Marag *et al.*, 2018).

Ammonia (NH_3) production: The bacterial isolates were cultured in 10 ml of peptone water in a test tube at 28°C for 2-3 days (gL^{-1}: peptone-10; sodium chloride-5; pH-7.2). Next, 0.5 ml of Nessler's reagent ($g100ml^{-1}$: mercuric chloride-10; potassium iodide-7; sodium hydroxide-16; water-100 ml; and pH-13) was added to each bacterial inoculum tube. A change in color from brown to yellow was considered a positive result, indicating the presence of ammonia produced by the bacteria (Cappuccino & Sherman, 1992).

Screening of endophytic bacteria for biochemical substances

Catalase activities: A small amount of bacterial culture was mixed with a drop of 3% H2O2 (30 µl) on a glass slide with an inoculating needle. Bubbles formation indicated, catalase activity (Eun-Kyung *et al.*, 2012).

Citrate utilization: The bacterial colony was streaked on slants of modified Simmon citrate agar and incubated at 28°C for 2-3 days (gL^{-1}: magnesium sulfate-0.2; ammonium dihydrogen phosphate-1.0; dipotassium phosphate-1.0; sodium citrate-2.0; sodium chloride-5.0; bromothymol blue-0.08; agar-15; and pH-6.8). Growth and a color change from green to deep blue indicated the positive result.

Evaluating enzymatic activity of bacterial isolates

Protease activity: To assess the protease activity of the bacterial isolate, a culture medium was utilized (gL^{-1}: yeast extract-2.5; skimmed milk powder-28; dextrose-1; Tryptone-5; and agar-15, with pH 6.8-7.0). The bacterial colonies were spot inoculated using autoclaved toothpicks and then incubated at 28°C for 2-5 days. A clear zone around the colony was considered indicative of enzymatic protease degradation (Chaiharn *et al.*, 2008).

Lipase activity: The bacterial isolates were cultured on a medium containing 1% sterilized Tween 20 and incubated at 28°C for 2-5 days (gL^{-1}: peptone-10; NaCl-5; $CaCl_2H_2O$-0.1; agar-16; pH-6.0). A clear zone around the bacterial colony indicated the presence of lipase activity (Hankin & Anagnostakis, 1975).

Pectinase activity: Pectinase activity was screened using a minimal medium modified with Pectin and incubated for 2 days at 28°C ($g\ L^{-1}$: ammonium sulfate-1; disodium phosphate-6; monopotassium phosphate-3; polygalacturonic acid (pectin)-5; agar-15 and pH-7). Flooding culture plates were observed in the pectin hydrolysis zones with an iodine solution ($g\ L^{-1}$: iodine-3 and potassium iodide-15).

Cellulase activity: To test for cellulose activity, bacterial isolates were grown on Carboxy-Methyl-Cellulose (CMC) agar plates (with the following composition per liter: dipotassium hydrogen phosphate-0.5 g; magnesium sulfate-0.25 g; carboxymethylcellulose-2 g; agar-15 g; gelatin-2 g; and pH-7). The plates were spot inoculated with the bacterial isolates and incubated for 3-5 days at 28°C. Afterward, Gram's iodine solution (made with 2.0 g of potassium iodide and 1.0 g of iodine in 300 ml of water) was flooded onto the plates for 3-5 minutes. A clear zone surrounding bacterial growth was a positive sign of cellulase production (Hendrick *et al.*, 1995).

Amylase activity: The bacterial isolates were inoculated on starch agar plates using a spot inoculation technique and incubated at 35°C for 72 to 96 hours (gL^{-1}: beef extract-3; soluble starch-10; agar-12, pH-7.5). After incubation, 6 ml of a 1% iodine-potassium iodide solution ($g100ml^{-1}$: iodine-0.34 and Potassium iodide-0.66) was added. A light zone around the bacterial colony was a positive result for the starch hydrolysis test (Krishnan *et al.*, 2012).

Esterase activity: The bacterial isolates were incubated at 28°C for 2-5 days on a culture medium containing 1% sterilized Tween 80 (gL^{-1}: peptone-10; NaCl-5; $CaCl_2H_2O$-0.1; agar-18; pH-7.4). The appearance of a clear zone around the colony indicated esterase activity (Castro *et al.*, 2014).

Asparaginase activity: Modified M9 agar medium supplemented with phenolic red at 0.009% was used for screening of asparaginase activity ($g\ L^{-1}$: L-Asparagine-10; $MgSO_4.7H_2O$-0.12; KH_2PO_4-3; NaCl-0.5; Na_2HPO_4-6; $CaCl_2.2H_2O$-0.001; and agar-20). The bacterial isolates were inoculated onto a culture medium and incubated at 37°C for 2 days. If a pink zone appeared around the colony, it was considered a positive result for asparaginase activity.

Heavy metal resistance test: The bacterial isolates were spot inoculated on LB agar medium containing heavy metals (mgL^{-1}: Pb-200; Cd-20; Zn-100; Cu-50; and Ni-20) and incubated at 28°C for 2-3 days. The growth of bacterial isolates indicated resistance to heavy metals (Sheng *et al.*, 2008).

Antibiotic resistance of the bacterial isolates: The bacterial isolates were spot-inoculated on LB agar medium that contained different antibiotics (mg ml^{-1}: streptomycin-20, kanamycin-20, ampicillin-100, or spectinomycin-20), and then incubated at 30°C for 7 days (Sheng *et al.*, 2008). The bacterial growth indicated a positive sign of antibiotic resistance.

Hydrogen Cyanide (HCN) production: The bacterial isolates were streaked on an LB culture medium containing 4.4 g L^{-1}glycines. The Whatman filter paper No.1 was dipped in a 0.5% picric acid solution and 2% sodium carbonate. It was then positioned on the lid of the plate and incubated at 28°C for 3 days. After the incubation period, the orange color was observed, indicating hydrogen cyanide production.

Antifungal assay: Antifungal activity was investigated on two phytopathogenic fungi: *Aspergillus niger* and *Alternaria brassicale*. The bacterial isolate was spot inoculated on the sides of PDA agar plates, and the test fungi were inoculated at the center using the Dual culture method. Control plates without bacterial isolates were also included. All plates were incubated at 30°C for 5 days, and any areas where bacterial colonies inhibited the fungal growth were considered a positive result.

Results

In this research study, 56 bacterial strains were isolated from different parts of *P. dactylifera* and subsequently evaluated for characteristics indicative of their potential to promote plant growth and serve as bio-inoculants (bio-fertilizers).

Isolation of bacterial endophytes: Fitty six bacterial strains were isolated, with the highest number of 41 isolates (73%) coming from the roots (PR), followed by 11 (20%) from the leaves (PL) and 4 (7%) from the stems (PS) (Fig. 1).

Morphological identification of endophytic bacteria: The bacterial isolates showed diverse morphological characteristics, as depicted in Fig. 2. The predominant colony shape was circular (84%), followed by irregular (16%), with no filamentous shapes observed (Fig. 2A). Microscopic analysis indicated that the majority (95%) of the strains were gram-positive (Fig. 2B). Colony sizes ranged from small to large, with large colonies constituting 29%, and both small and punctiform colonies making up 27% each, while moderate-sized colonies accounted for 18% (Fig. 2C). Most strains exhibited raised (41%) and flat (23%) elevations (Fig. 2D). Colony colors varied, including white, creamy, creamy white, orange, and yellow, with creamy white being the most common (59%) (Fig. 2E). The most frequently observed colony margin was entire (84%) (Fig. 2F).

Growth characteristics of endophytic bacteria under different conditions: The average culture densities of the bacterial isolates under salt tolerances of 1, 3, and 5% were 1.10±0.22, 0.79±0.22, and 0.57±0.20, respectively, as shown in Fig. 3A. Under varying pH levels, the average culture densities were 0.18±0.05 at pH 4, 0.89±0.20 at pH 7, and 0.19±0.05 at pH 10 (Fig. 3B). For temperature conditions, the average culture densities were 0.89±0.19 at 30°C and 0.70±0.23 at 40°C, while the lowest average density of 0.17±0.05 was recorded at 15°C, as depicted in Fig. 3C.

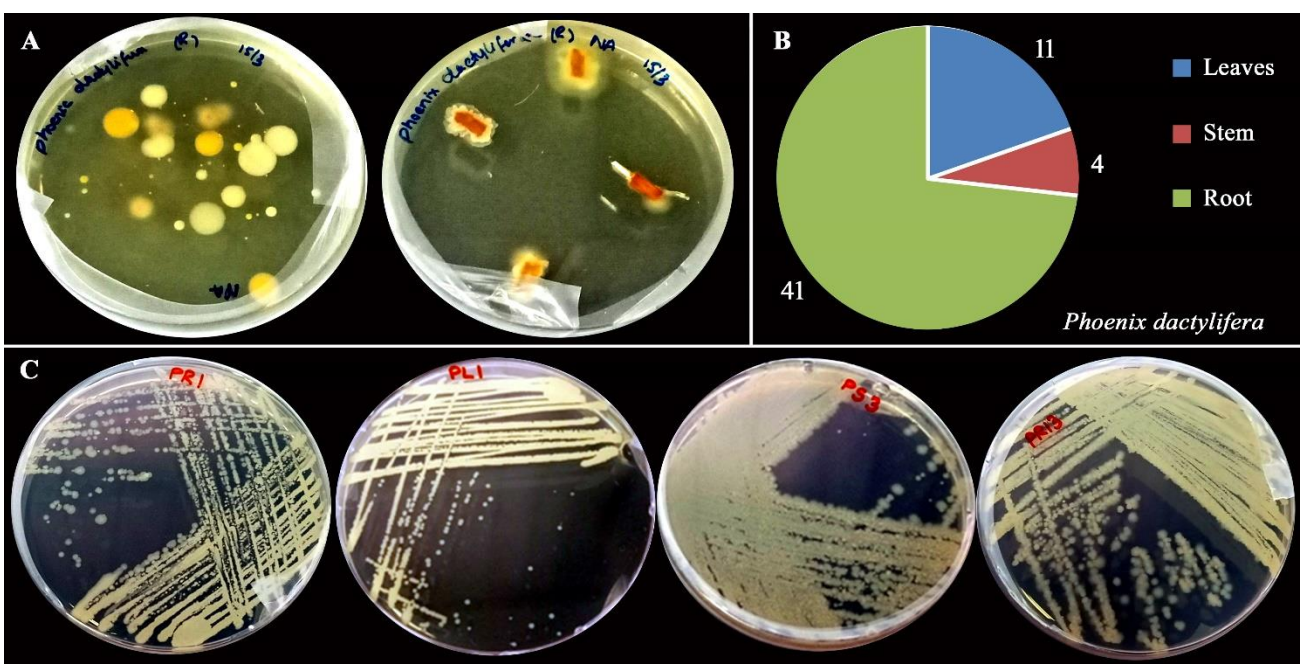

Fig. 1. Isolation of bacterial endophytes: A, plate showing growth of bacterial strains; B, the total number of bacterial isolates from the leaves, stems and roots of *Phoenix dactylifera*; C, streaking was performed for the isolation and purification of bacterial strains.

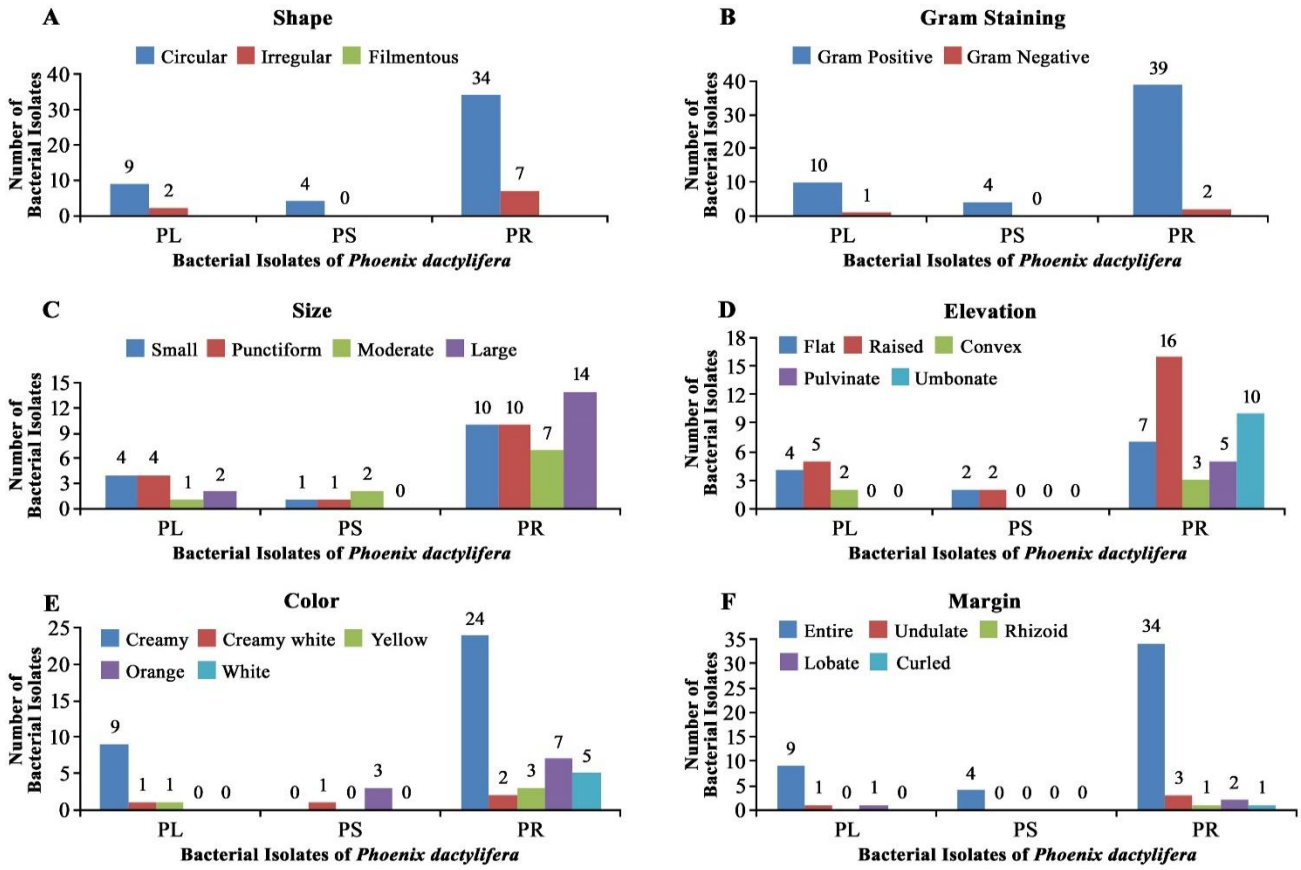

Fig. 2. Morphological identification of bacterial isolates of *Phoenix dactylifera*, based on: A, shape; B, gram staining; C, size; D, elevation; E, color; F, margin.

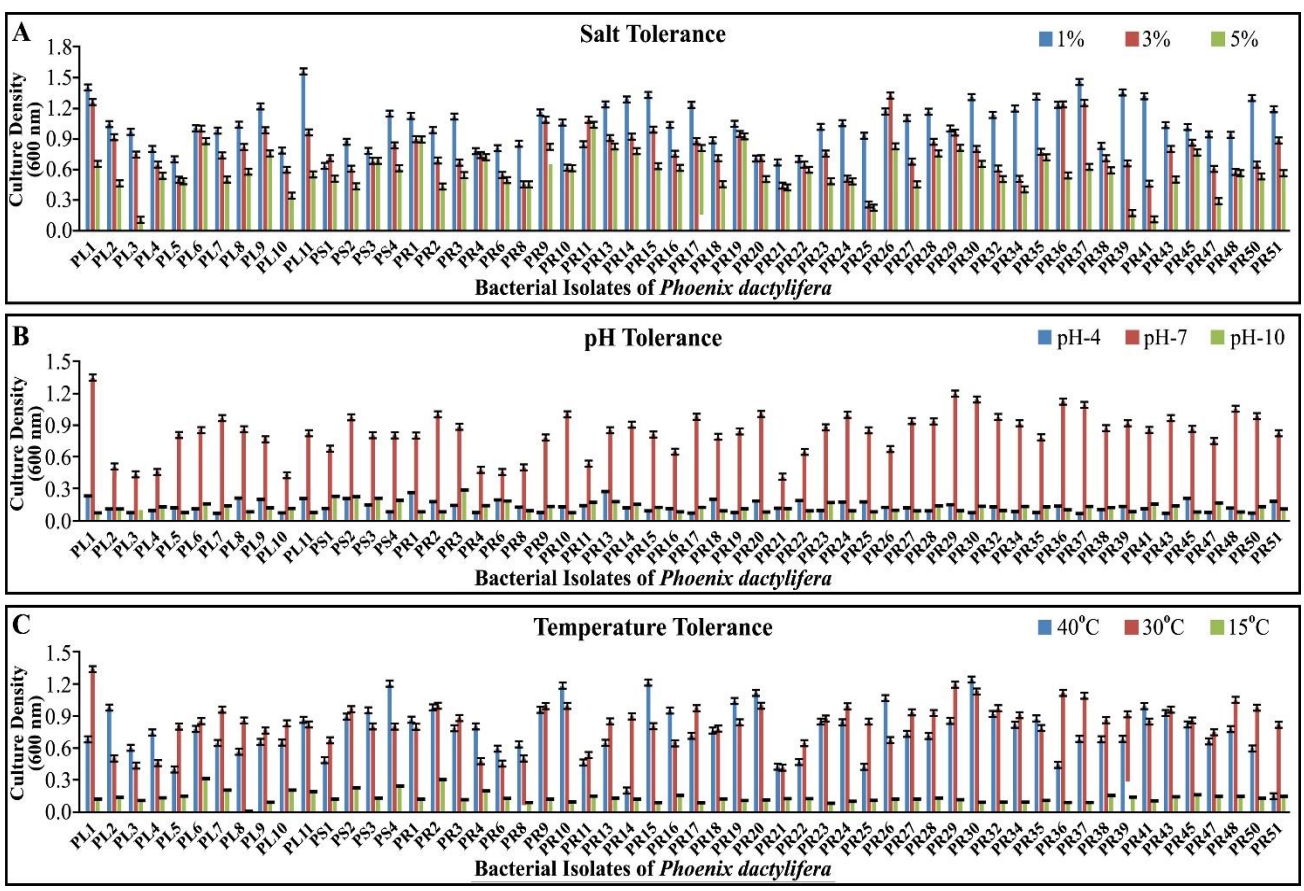

Fig. 3. Growth of bacterial isolates of *Phoenix dactylifera,* under different NaCl concentrations, pH levels, and temperatures.

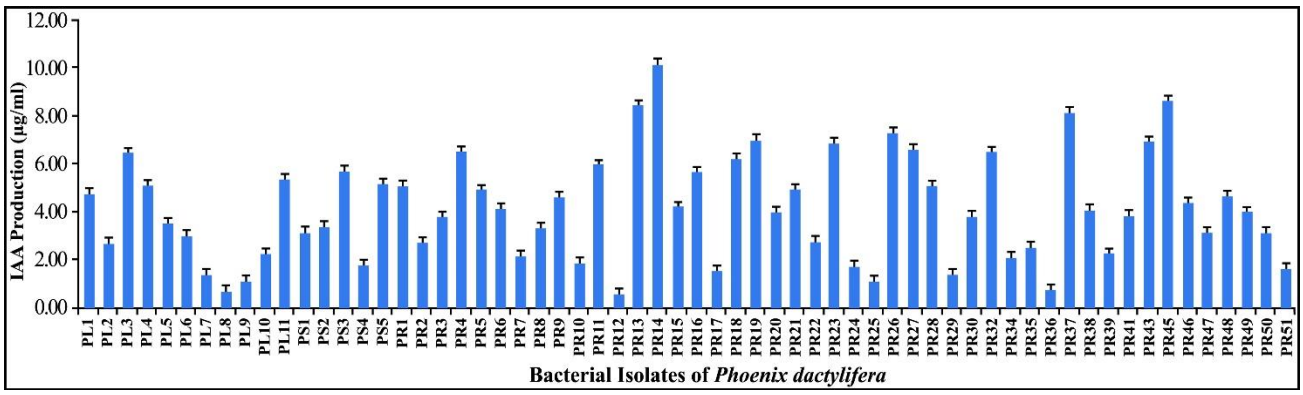

Fig. 4. IAA productions by bacterial isolates of *Phoenix dactylifera.*

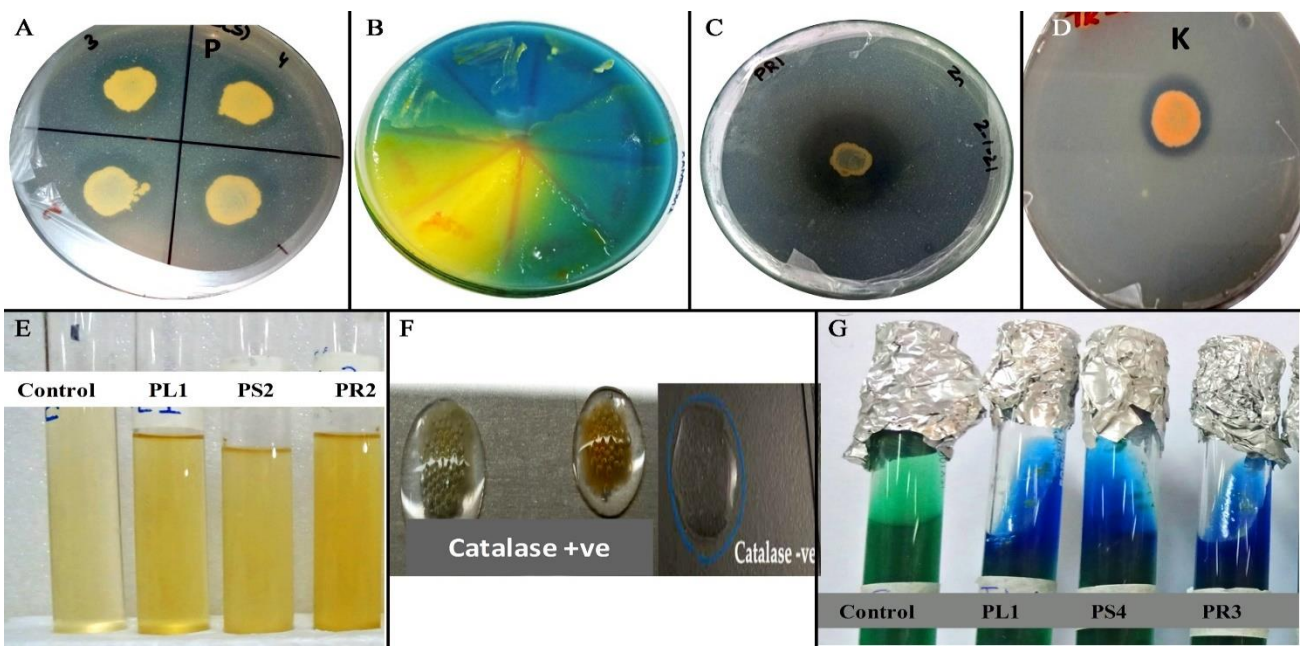

Fig. 5. Plant growth-promoting traits of bacterial isolates: A, Phosphate solubilization; B, Nitrogen fixation; C, Zinc solubilization; D, Potassium solubilization; E, Ammonia production; F, Catalase activity; G, Citrate utilization.

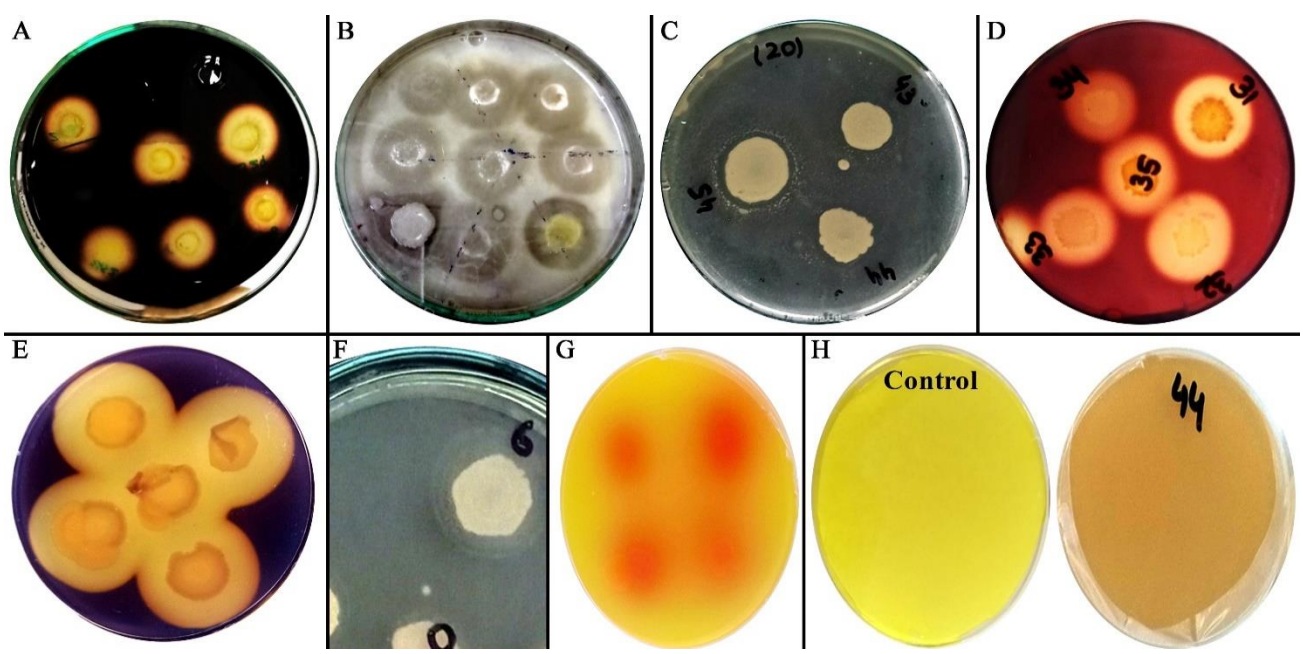

Fig. 6. Bacterial isolates exhibiting: A, Amylase activity; B, Protease activity; C, Lipase activity; D, Pectinase activity; E, Cellulase activity; F, Esterase activity; G, Asparaginase activity; H, Hydrogen Cyanide production.

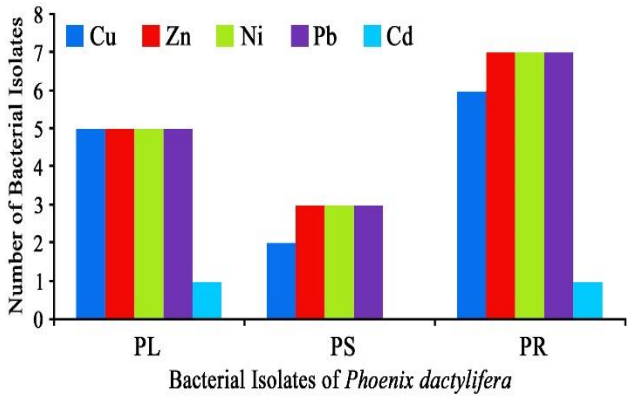

Fig. 7. Bacterial isolates showing resistance against heavy metals.

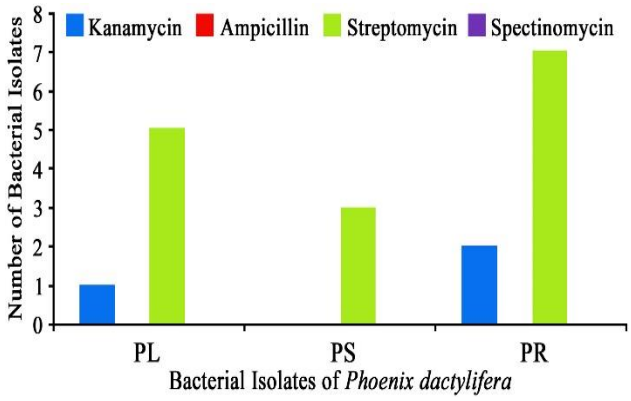

Fig. 8. Bacterial isolates showing resistance against antibiotics.

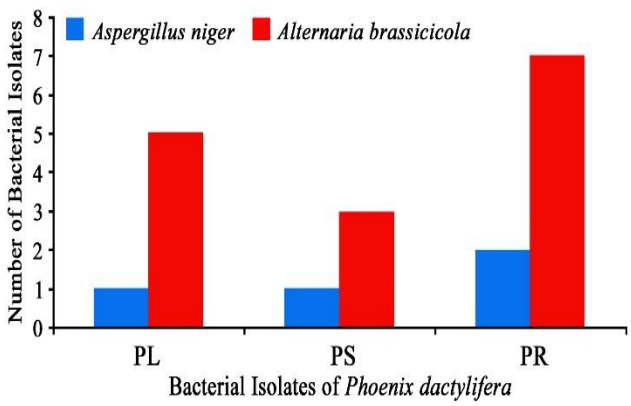

Fig. 9. Antifungal activity of bacterial isolates against *Aspergillus niger* and *Alternaria brassicicola.*

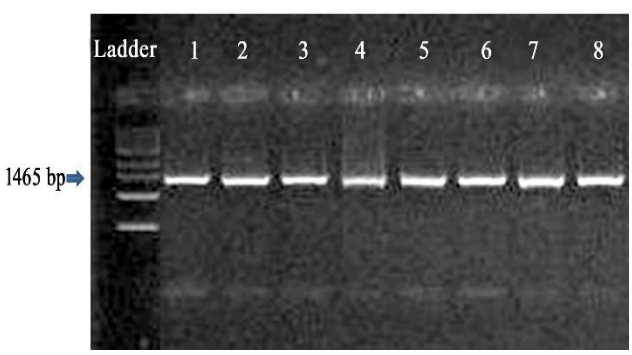

Fig. 10. The PCR amplification of the 16S rRNA gene of bacterial isolates is Shown, Lane 1 contains a 1 kb DNA ladder, while lanes 2 to 8 show amplified DNA fragments of approximately 1465 bp.

Molecular identification of bacterial isolates: Amplification of the 16S rRNA gene of the 15 chosen isolates was performed using universal primers 27F and 1492R, resulting in a product size of approximately 1500bp (Fig. 10).

Biochemical and enzymatic characteristics of the bacterial isolates: Most of the isolates (99%) were positive for catalase activity (Fig. 5F). A total of 30 (54%) bacterial isolates demonstrated a positive outcome, as indicated by the emergence of a color change from green to blue and the accompanying growth of citrate production (Fig. 5G). A total of 39(70%) bacterial isolates were found to be positive for amylase activity (Fig. 6A). Based on qualitative tests a total of 44 (79%) isolates produced protease enzyme by developing a clear zone on the skimmed milk agar medium (Fig. 6B).

Selection of best-performing bacterial isolates: Based on the plant growth-promoting properties (IAA production, phosphate solubilization, potassium and zinc solubilization, nitrogen and ammonia production, amylase, catalase, and protease activities), out of 56 bacterial isolates, 15 were selected for further analysis.

Selected bacterial isolates screened for biocontrol properties: Bacterial isolates were spot inoculated on media agar plates co-polymerized with 1% of the substrate Tween 20 lauric acid esters. Out of 15 isolates, 14 isolates show lipase activity (Fig. 6C). The bacterial isolates were examined for pectinase production using a qualitative test. Out of 15 isolates, 9 showed positive pectinase activity (Fig. 6D). All 15 isolates produced cellulose enzymes with the development of a halo zone on the CMC agar medium (Fig. 6E). For Esterase activity, bacterial isolates were spot inoculated on media agar plates co-polymerized with 1% of the substrate Tween 80 oleic acid esters. A total of 2 bacterial isolates displayed positive enzymatic activity (Fig. 6F). The production of the asparaginase enzyme was confirmed by observing a color change from yellow to pink in the M9 agar medium surrounding the colony after 2-5 days. All 15 isolates were found to be capable of producing the asparaginase enzyme (Fig. 6G). The change in color from light yellow to orange-brown after 2-3 days of incubation indicated a positive result for HCN production. All 15 bacterial isolates produced HCN (Fig. 6H).

Heavy metal resistance test: All 15 bacterial strains were resistant to zinc, nickel, and lead while resistance to copper and cadmium showed a mixed trend. A total of 13 isolates were resistant to copper while 2 isolates were resistant to cadmium as shown in Fig. 7.

Antibiotic resistance of the bacterial isolates: Interestingly, no strain was observed to be resistant to ampicillin and spectinomycin. Streptomycin resistance was common among all isolates, whereas 3 isolates were resistant to kanamycin (Fig. 8).

Antifungal assay: The dual culture assay method was used to assess the antifungal activity of the bacterial isolates against phytopathogenic fungi (Fig. 9). All 15 isolates were effective against *Alternaria brassicicola*, while 4 isolates were effective against *Aspergillus nigar*.

Table 1. Identification of bacterial isolates from *Phoenix dactylifera* with accession number by NCBI.

Strain ID	Number Of Nucleotides (16SrRNA gene)	Accession number of 16SrRNA gene	Closely related validly published taxa	Similarity % Age of 16sr RNA gene sequence with closely related species	Cover age %	Number of closely related species having >98.6% similarity of 16Sr RNA gene sequence
PL1	1427	OR394183	*Bacillus safensis* subsp. *safensis* FO-36b^T (ASJD01000027)	99.72	100	9
PL2	1409	OR394184	*Bacillus paranthracis*Mn5^T (MACE01000012)	99.86	100	34
PL3	1400	OR394186	*Bacillus paramycoides*NH24A2^T (MAO101000012)	99.50	100	34
PL4	1416	OR394187	*Bacillus paramycoides* NH24A2^T (MAO101000012)	99.93	100	34
PL11	1421	OR394188	*Bacillus safensis subsp. safensis* FO-36b^T (ASJD01000027)	100	100	9
PS1	700	OR430871	*Bacillus tropicus*MCCC 1A01406 (NR_157736.1)	88.24	39	0
PS3	1455	OR394190	*Bacillus safensis* subsp. *safensis* FO-36b^T(ASJD01000027)	99.24	100	8
PS4	1415	OR394253	*Bacillus paranthracis* Mn5^T (MACE01000012)	99.16	100	24
PR1	1432	OR394252	*Bacillus safensis* subsp. *Safensis* FO-36b^T (ASJD01000027)	99.09	100	5
PR13	1462	OR394189	*Bacillus safensis* FO-36b (NR_041794.1)	92.68	97	0
PR19	496	OR437953	*Peribacillus acanthi*L28 (NR_179899.1)	88.68	61	0
PR23	995	OR437966	*Bacillus aerius*24K (NR_118439.1)	93.19	82	0
PR26	1422	OR394239	*Bacillus anthracis* ATCC 14578 (NR_041248.1)	97.20	90	0
PR37	1422	OR394240	*Bacillus anthracis*ATCC 14578 (NR_041248.1)	97.59	90	0
PR45	1412	OR394241	*Haemophilus parainfluenzae*ATCC 33392 CIP 102513 (NR_116168.1)	98.09	99	0

The sequenced data from the isolated strains displayed similarities to species previously documented in the NCBI GenBank database (Table 1). The outcomes of the molecular characterization highlighted that *Bacillus* was the dominant genus within the characterized bacterial strains.

Discussion

Bacterial endophytes are integral to improving plant growth and resilience by enhancing nutrient uptake and protecting against environmental stress. In this study, a total of 56 culturable bacterial strains were isolated from the leaves, stem, and root of *Phoenix dactylifera*, displaying diverse morphologies. Notably, 95% of the isolates were Gram-positive, and 84% exhibited a circular colony shape. This predominance of Gram-positive bacteria aligned with previous findings of Sgroy *et al*., (2019), who reported 68.9% Gram-positive bacteria in the root of *Prosopis strombulifera*. Similarly, Amrullah *et al*., (2018) noted creamy wet pigmentation, round forms, and Gram-positive bacteria among endophytes isolated from red betel roots. The colony sizes in this study ranged from small to large, with 41% of strains showing raised elevations, which is consistent with Padder *et al*., (2017), who observed convex elevations dominating among isolates from *Brassica rapa L.*. Additionally, 23% of colonies in our study exhibited flat elevations, resembling findings by Amrullah *et al*., (2018). Colony pigmentation was also consistent across studies, with creamy white being the most common color, observed in 59% of strains here and similarly reported in studies by Chauhan & Singh (2023). These results emphasize a broad morphological diversity among isolates, which aligns with observations in similar studies across various host plants, highlighting conserved traits in bacterial endophyte populations.

Bacterial strains exhibit resistance to abiotic conditions such as salinity, pH, and extreme temperatures can indeed be valuable bioresources for enhancing agricultural output. These resilient bacteria can potentially be employed in agricultural practices to improve crop production and protect plants from adverse environmental conditions. The overall decrease in culture density of bacterial isolates under 3% and 5% salt stress levels were 31% and 53% compared to 1% salt stress level. These results provide valuable insights into the response of bacterial isolates' culture density to different salt concentrations, highlighting their growth characteristics and adaptability under varying saline conditions. Similar findings with isolates from various *Acacia* tree species have been reported by several researchers (Odee *et al.*, 1997; Zerhari *et al.*, 2000; Fterich *et al.*, 2012). Many strains that exhibit high resistance to salt have been isolated from *Acacia* trees in India, Pakistan, and Morocco (Kumar *et al.*, 1999). Relatively higher (89%) culture densities of bacterial isolates were obtained at pH 7 as compared to pH 10 and pH 4 confirming the ability of some bacterial isolates to survive both in alkaline and acidic soil by tolerating a wide range of pH. Similar findings were reported by Rehman & Nautiyal (2002) and Fetrich *et al*., (2012), demonstrated that certain bacteria that form nodules on legumes could, endure alkaline pH levels of up to 12. The results of this study indicated that the bacterial isolates preferred moderate temperatures (around 30°C) for optimal growth and proliferation, while their growth may be reduced at both higher (40°C) and lower (15°C) temperatures. This suggests that these bacterial isolates may have difficulty in surviving or maintaining their growth at temperatures exceeding 40°C. Higher temperatures can have detrimental effects on bacterial metabolism, cellular processes, and overall viability,

leading to a decline in culture densities. These findings emphasize the high elevated sensitivity of these bacterial isolates to temperatures and highlight the importance of temperature regulation for their survival and growth. Zerhari *et al*., (2000) demonstrated that 48 isolates derived from *Acacia species* could thrive at 40°C.

All bacterial isolates exhibited indole-3-acetic acid (IAA)production, ranging from 0.60 to 10.13 µg ml^{-1} with an overall average of 4.23 ± 2.19 µg ml^{-1}. These results were similar to the investigation performed by Ahemad and Khan, 2010; Jasim *et al.*, 2013; Ullah *et al.*, 2018; Long *et al*., 2008. Similar conclusions were drawn by Khalid *et al*., (2004) who strongly attributed plant growth promotion to the IAA-producing bacteria by improving nutrient uptake from soil. According to the findings of this study, 41% of the bacterial isolates tested were able to solubilize phosphate. Inorganic phosphate is solubilized, most commonly, by the dissolution of mineral compounds such as protons, carbon dioxide, organic acids, hydroxyl ions, and siderophores (Tian *et al.*, 2021; Olanrewaju *et al.*, 2017). Microorganisms can solubilize phosphate and enhance soil quality(Alori *et al.*, 2017). According to this study, 20% of the isolates were very effective in solubilizing potassium, which is essential for increasing agricultural productivity and output. Numerous endophytes produce an enzyme that can solubilize potassium, which is an essential nutrient for plant growth (Bahadur *et al.*, 2016; Tsegaye *et al.*, 2019).

In this study, majority of bacterial isolates (82%) demonstrated growth when streaked on the Jensen medium. Some endophytic bacteria possess both nitrogen fixation and denitrification genes (Straub *et al.*, 2013; Gourion *et al.*, 2014; Sulistiyani & Meliah, 2017; Kayasth *et al.*, 2014). Microorganisms are known to play a significant role in the solubilization of zinc in the soil (Fasim *et al.*, 2002).In this study, 87% of the bacterial isolates were capable of solubilizing zinc oxide in a Tris-minimal medium. According to our findings, 93% of the isolates were capable of generating ammonia. Ammonia is important for plant growth as it provides nitrogen to plants (Yadav *et al.*, 2010; Ahemad & Khan, 2010). Most of the isolates (96%)showed positive results for catalase activity. Bacteria-producing catalase enzymes possess high resistance to various environmental, mechanical, and chemical stresses (Kumar *et al.*, 2012).In this study, 53%of the isolates showed citrate utilization indicating that these bacterial isolates possessed the ability to utilize citrate as a nutrient. Most of the isolates (70%) produced amylase and 78% produced protease enzyme. Microorganisms utilize hydrolytic enzymes to penetrate the plant and regulate phytopathogens, as well as promote stress tolerance (Mutungi *et al.*, 2022).

Our findings aligned with previous studies, which also reported that endophytes possess multiple plant-promoting traits such as phosphate solubilization, the production of phytohormones, cell wall degrading enzymes, and nitrogenase activity (Borah & Thakur, 2020). The 15 best-performing isolates were selected for additional testing and screening. All isolates were positive for HCN production. Hydrogen cyanide (HCN) and ammonia are volatile compounds known for their antimicrobial activity, making them effective biocontrol agents against plant diseases (Brimeconbe *et al.*, 2001; Rijavec & Lapanje, 2016). Most of the isolates (93%) tested positive for lipase, 60% for pectinase, and 13% for esterase activity. In another study, Theantana *et al*., (2007)investigated the production of asparaginase by endophytes isolated from a Thai medicinal plant. The results of this study revealed that asparaginase and cellulase activity was positive in all the bacterial isolates. Cellulase and pectinases are important for deeper interaction and to protect the plant host (Reinhold-Hurek & Hurek, 2011).

These selected isolates were resistant to streptomycin, and 3 isolates were resistant to kanamycin according to Sheng *et al*., (2008), two strains of bacteria,*P. fluorescens* G10 *and Microbacterium* sp. G16 were found to be resistant to antibiotics kanamycin, ampicillin, and spectinomycin, as well as heavy metals such as Cu, Zn, Ni, Pb, and Cd. According to the result of this study, these bacterial strains were also resistant to zinc, nickel, and lead while resistance to copper and cadmium was 87% and 13 %respectively and similar isolates were effective against *Alternaria brassicicola*, while 4 were effective against *Aspergillus nigar*, demonstrating that endophytic microbes can inhibit pathogen growth by releasing different enzymes, metabolites especially protease, lipase, esterase, pectinase, cellulose, HCN, siderophore, chitinase, jasmonic acid and also salicylic acid (Kumar *et al.*, 2019).

In this study, 15 best-performing bacterial isolates were further analyzed using 16S rRNA gene sequencing. The molecular characterization revealed that the predominant genus among the characterized strains was *Bacillus*. Tsipinana *et al*., (2024) found that *Bacillus* species, such as *B. licheniformis* and *B. velezensis*, promoted seedling growth in *Lessertia frutescens* through their plant growth-promoting properties, including enhanced plant height and bio-agent potential. Singh *et al*., (2024) also highlighted the significant role of endophytic *Bacillus* species in alleviating various non-biological stressors like salinity, drought, and heavy metals, emphasizing their potential to enhance agricultural productivity, particularly under changing climatic conditions. These studies reinforced *Bacillus* as a key genus for sustainable agriculture and plant growth enhancement. Many *Bacillus* species are known to have plant growth-promoting capabilities, including the ability to produce phytohormones, solubilize nutrients, and induce systemic resistance in plants (Figuedireda *et al.*, 2009; Krishnan *et al.*, 2012). *Bacillus* species possess a diverse set of plant growth-promoting (PGP) characteristics, including solubilizing potassium, zinc, and phosphate, producing antibiotics, fixing nitrogen, producing indole acetic acid (IAA), generating hydrogen cyanide (HCN) and ammonia, exhibiting stress tolerance, and secreting enzymes (Medeiros *et al.*, 2011).

Conclusion

In this study, 56 bacterial strains obtained from different parts of *Phoenix dactylifera* were successfully isolated, identified, and evaluated for their plant growth-promoting (PGP) properties. Overall, 27% of bacteria isolates performed better in terms of PGP properties, antibacterial, antifungal activity, and heavy metal resistance tests. It can be concluded that the endophytic bacteria from *Phoenix dactylifera* have significant potential as biofertilizers and biocontrol agents. Their use could potentially reduce reliance on chemical fertilizers and pesticides, offering a more environment friendly and health-conscious alternative to traditional agricultural practices.

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