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Phylogenetic Analysis of Bacterial Isolates Recovered from Salt-affected Soils of Haryana & Punjab, India

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Authors' contributions

This work was carried out in collaboration between both authors. Author PBK designed the study and wrote the protocol. Author SKS managed the analyses of the study, managed the literature searches and wrote the paper. Both authors read and approved the final manuscript.

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ABSTRACT

Excessive saline/alkaline conditions present a significant challenge to the environment and ecology, impacting yield, plant growth, and soil health. This study focuses on isolating and characterizing halophilic bacteria from salt-affected areas in Haryana and Punjab, India. Morphological, biochemical, and molecular analyses were conducted to assess their potential as plant growth-promoting rhizobacteria (PGPR) for mitigating salt stress in salt-affected soils. Four bacterial strains, identified as HR3-PM, PB01-KB, PB-424, and PB-466, were isolated and characterized. Morphological and biochemical assays revealed diverse traits among the isolates, including phosphate solubilization, indole-3-acetic acid (IAA) production, and ammonia excretion. Molecular identification via 16S rRNA sequencing confirmed their taxonomic classification and revealed close homology to known bacterial species such as *Klebsiella aerogenes*, *Pseudomonas mosselii,*

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Lysinibacillus acetophenoni, and *Pseudomonas stutzeri*. Phylogenetic analysis provided insights into their evolutionary relationships. These salt-tolerant bacteria exhibit promising PGPR activities, suggesting their potential for sustainable agriculture and soil remediation practices in salt-affected soils. Harnessing their abilities could offer cost-effective and environmentally friendly solutions to mitigate soil salinity, enhance plant productivity, and contribute to global environmental stresses. Further research is warranted to fully understand and harness the biotechnological potential of these halophilic bacteria in salt-affected ecosystems, paving the way for a more sustainable environmental future.

Keywords: Halophilic bacteria; Plant Growth-Promoting Rhizobacteria (PGPR); salt-affected areas; salt stress; soil salinity; sustainable agriculture.

1. INTRODUCTION

Salt-affected soil causes global crop losses and affects soil properties [1]. An estimated $11 billion less in income every year as a result of salinity problems. With 9.5 billion people on the planet by 2050, farming saline lands will be essential [2,3]. A study from the Western Yamuna and Bhakra canal commands in Haryana, India, found that irrigation-induced waterlogging and salinity drastically reduced plant product yields, leading to dismal farm incomes and a decrease in farm employment [4]. Salt accumulation in soil threatens food security, impacting physiological processes like reproductive physiology, flowering, fruiting, and soil processes such as decomposition, respiration, denitrification, and biodiversity [5,6]. Several methods have been developed to mitigate the harmful effects of salt stress on plants, including genetic modification and the use of PGPR [7].

The use of halophilic bacteria aids in the restoration of soils damaged by salt by directly promoting plant development [8]. Research indicates that abiotic stress can be mitigated by isolating bacteria from severe environmental stressors [9]. PGPR strains are gaining interest as potential biocontrol agents for suppressing plant pathogens and inducing disease resistance in plants. Salt-tolerant PGPR (ST-PGPR) has been reported to ameliorate salt stress in the plant by direct and indirect mechanisms [10,11 & 12]. Direct mechanisms include altered nutrition through the provision of fixed nitrogen; iron through siderophores; soluble phosphate (P) and zinc (Zn); the production of phytohormones such as indole acetic acid (IAA), cytokinin, and gibberellins; or by the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. Several soil/plant-colonizing microbiomes that contain the vital enzyme ACC deaminase can lessen the harmful effects of excessive ethylene levels [13,14]. Indirect mechanisms include the suppression of pathogens through the action of siderophores, and the production of antibiotics and extracellular hydrolytic enzymes [15,16]. Microorganisms employ different strategies for stress tolerance [17]. Important actinomycetes belonging to the genus Streptomyces are thought to create a broad range of physiologically energetic chemicals, some of which have anticancer, anti-infective, and medicinal benefits [54].

Evidence suggests secondary metabolites are involved, despite reports claiming microbes alleviate abiotic stress by triggering basic metabolisms (plant growth, food uptake, photosynthesis, and antioxidant enzymes [18]. Some secondary metabolites such as flavonoids, phytoalexins, phenylpropanoids, and carotenoids have been documented in stressed plants inoculated with microorganisms [19,20]. Both Gram-positive and Gram-negative bacteria produce siderophores under iron deficiency, [21, and 22]. Enzymes derived from extremophiles, called extremozymes, that function under harsh conditions not suitable for conventional enzymes generated by these bacteria are extremely useful in industry and hold great industrial importance [23]. Promising biotechnological uses for halophiles include the production of pigments, enzymes, surfactants, decolorization of textile dye effluents, metal degradation, and pollution remediation from saline water. Recent research on halophilic fungi in hypersaline environments suggests that there is a wide variety of fungal species present. In 2000, research was conducted on halophilic fungi, with an emphasis on their morphological and molecular properties in a saline environment [53]. Kushwaha, et al. [24] reported that root-colonizing bacteria produce phytohormones that alleviated salinity-induced dormancy and elicited seedling growth. Moreover, (Kumar, et al. [25] showed that *Bacillus* and *Pseudomonas sp.* produced

siderophores, indole acetic acid (IAA), and solubilized phosphates to boost growth in stressed plants. ACC-deaminase-containing microorganisms inhibit ethylene synthesis, enhancing root growth [26,27]. Lowered ethylene levels resulted in root growth and improved the survival of stressed plants [28]. Microorganisms employ different strategies for stress tolerance [29]. Suitable management practices for salt-affected soils are different than for normal soils. Therefore, the present study aimed to isolate and screen halophilic strains from extreme environments and investigate the effects of physiological conditions including temperature, pH, and sodium chloride concentrations on strain growth. Isolated halophilic bacterial strains can alleviate the salt-affected soils.

2. MATERIALS AND METHODS

2.1 Study Areas

The study focuses on salt-affected soil samples from Bathinda (Punjab) and Fatehabad (Haryana) districts. Fatehabad, spanning 2520 km², in the Indo-Gangetic basin, experiences a tropical climate with brackish subsurface water, relying on Bhakra and Western Yamuna canals for irrigation. In southern Punjab, Bathinda features sandy soil with sporadic eastward-leaning sand dunes.

2.2 Collection of Soil Sample

Soil samples were collected from salt-affected soils in Ratiya, mixed woodland in Fatehabad, Haryana, and agricultural ground in Bhatinda, Punjab. Soil samples were obtained from various locations in sterile plastic vials and transported to a soil microbiology laboratory for storage at 4°C.

2.3 Morphological Characterization

Colony morphology, cell shape, and Gram reaction as per the standard procedures of Anonymous (1957) and Barthalomew and Mittewer [30,31]. Colony morphology as per Cappuccino and Sherman [32].

2.4 Biochemical Characterization

Assay for IAA [33], Phosphorus solubilization [34], Ammonia excretion [35], dextrose fermentation test by Hugh and Leifson [36], mannitol fermentation test, and lactose fermentation test [37,38].

2.5 Phyogenetic Identification Based on the Amplification of 16S rRNA

For amplification of the 16S rRNA gene, Genomic DNA was extracted by using TE buffer. Pure bacterial cultures were suspended in a centrifuging tube containing a buffer solution. After heating at 95°C for 10 minutes and centrifuging at 10,000 rpm for 5 minutes, the supernatant was used as template DNA for 16S rRNA amplification. Universal primers were used for the amplification of genes. The amplified product was purified and sequenced. Sequences obtained were analyzed and identified using BLAST- Basic Local Alignment Search Tool [42] and were compared against bacterial 16S rRNA sequences available on the NCBI database. The sequences were aligned followed by the construction of a neighbor joining the phylogenetic tree by using Clustal W using MEGA 10 [43].

3. RESULTS

3.1 Morphological and Biochemical Characterization of Isolates

Salt-tolerant bacteria isolated from salt-affected soil were characterized and studied for their morphological, biochemical, and physiological characteristics.

3.2 Morphological Characters

Characterization of bacterial colonies for colour, form, margin, elevation, etc. was performed by Gram staining, and cell wall morphological studies under a microscope were also performed (Table 1). Four isolates, namely HR3-PM, PB01-KB, PB-424, and PB-466, were examined for colony morphology and Gram stain characteristics. HR3-PM displayed regular colonies with a cream colour, and undulated margin, and was Gram-negative. PB01-KB exhibited regular, yellow colonies with an entire margin and raised elevation, also identified as Gram-negative. In contrast, PB-424 showed irregular, beige colonies with an entire margin and raised elevation, being Gram-positive. Finally, PB-466 had irregular, brownish colonies with an entire margin, raised elevation, and was Gram-negative. These details provide valuable insights into the diverse characteristics of these bacterial isolates.

Table 1. Morphological characterizations of halophilic bacterial strains

S. No	Isolates	Colony morphology				Gram stain
		Form	colour	Margin	Elevation	
1	HR3-PM	Regular	Cream	Entire	Undulated	Gram-negative
2	PB01-KB	Regular	yellow	Entire	Raised	Gram-negative
3	PB-424	Irregular	Beige	Entire	Raised	Gram-positive
4	PB-466	Irregular	Brownish	Entire	Raised	Gram-negative

3.3 Biochemical Characteristics

The isolates HR3-PM, PB01-KB, PB-424, and PB-466 were evaluated for their characteristics (Table 2). HR3-PM showed a negative result for indole-3-acetic acid (IAA) production but exhibited positive traits for phosphate solubilization, ammonia excretion, and utilization of dextrose and lactose, while it did not utilize mannitol. PB01-KB demonstrated positive IAA production and phosphate solubilization but lacked ammonia excretion capability. However, it efficiently utilized dextrose and lactose while not utilizing mannitol. PB-424 displayed positive results for all tested traits, including IAA production, phosphate solubilization, ammonia excretion, and utilization of dextrose, lactose, and mannitol. Similarly, PB-466 also exhibited positive outcomes for IAA production, phosphate solubilization, ammonia excretion, and utilization of dextrose and lactose, but it did not utilize mannitol. In another study by Yang, et al. [39], the *Klebsiella aerogenes* Wn strain was examined and the maximum dissolved phosphate was up to 701.36 mg/L by the strain Wn [35]. For seven days, the cultivation is carried out at 30 °C. According to Valenzuela-Aragon, et al. [40]; and Zuluaga, et al. [41], distinct bacterial isolates solubilize phosphate when clear zones appear surrounding their colonies.

3.4 Molecular Characterization

3.4.1 PCR amplification and sequencing of 16S rRNA gene

Isolated DNA strains (HR3-PM, PB01-KB) were investigated for 16S rRNA gene amplification. Its quality was evaluated on 1.0 % agarose gel, and a single band of high-molecular-weight DNA has been observed. The fragment of 16S rRNA gene was amplified by 16SrRNA-F and 16SrRNA-R primers. A single discrete PCR amplicon band of 1500 bp (base pair) was observed when resolved on agarose gel (Fig. 1). Contaminants were eliminated by purifying the PCR amplicon. Using the 16SrRNA-F and 16SrRNA-R primers, a forward and reverse DNA sequencing reaction of the PCR amplicon was performed on an ABI 3730xl Genetic Analyzer utilizing the BDT v3.1 Cycle sequencing kit to produce the gene sequence. Using aligner software, the consensus sequence of the 16S rRNA gene was produced from forward and reverse sequence data. The obtained sequences of both genes were compared with the available nucleotide sequences in NCBI using the BLAST- Basic Local Alignment Search Tool.

3.4.2 Phylogenetic characterization of isolated bacterial strains

Phylogenetic characterization of the salt-tolerant PGPR isolates was based on PCR amplification of 16S rRNA gene sequences. The bacterial isolates HR3-PM, PB01-KB, PB424, and PB466 were further selected for molecular characterization based on their promising salt tolerance and plant growth-promoting properties. The 16S rRNA gene of the isolates HR3-PM, PB01-KB, PB-466 and PB-424 were successfully amplified using PCR, and approximately 1500 bp of the amplified products were sequenced (Fig. 1). The 16S rRNA Gene sequence of these isolates was compared with the 'nr' database of NCBI GenBank database of NCBI using BLAST-Basic Local Alignment Search Tool (1990) after that, the algorithm builds a phylogenetic guide tree using the neighbor-joining approach and midpoint rooting. This phylogenetic tree is utilized to produce a global alignment and acts as a rough model for clades that frequently share insertion and deletion characteristics. The first ten sequences were chosen based on the maximum identity score, and they were aligned using the multiple alignment software Clustal W. Distance matrix [44]. The BLAST-N comparison of the searched sequences in the NCBI nucleotide database revealed HB3-PM displayed a striking 99.86% resemblance to *Klebsiella aerogenes* strain NBRC 13534 (Table 4), with a maximum score 2697 whereas PB01-KB closely mirrored *Pseudomonas mosselii* CFNL 90-83 with 99.80% homology with maximum score 2724 (Table 3). The BLAST-N comparison of the searched sequences in the

NCBI nucleotide database revealed PB-424 displayed a striking 98.81% resemblance to *Lysinibacillus acetophenone* strain JC23 (Table 5) with a maximum score of 2549 whereas, PB-466 closely mirrored *Pseudomonas stutzeri* ATCC 17588= LMG 11199 WITH 99.93% homology with maximum score 2647 (Table 6).

Table 2. Biochemical characterization of halophilic bacterial strains

S. No	Isolates	IAA	P-Solubilization	Ammonia excretion	Dextrose	Lactose	Mannitol
1	HR3-PM	-ve	+ve	+ve	+ve	+ve	-ve
2	PB01-KB	+ve	+ve	-ve	+ve	+ve	-ve
3	PB-424	+ve	+ve	+ve	+ve	+ve	+ve
4	PB-466	+ve	+ve	+ve	+ve	+ve	-ve

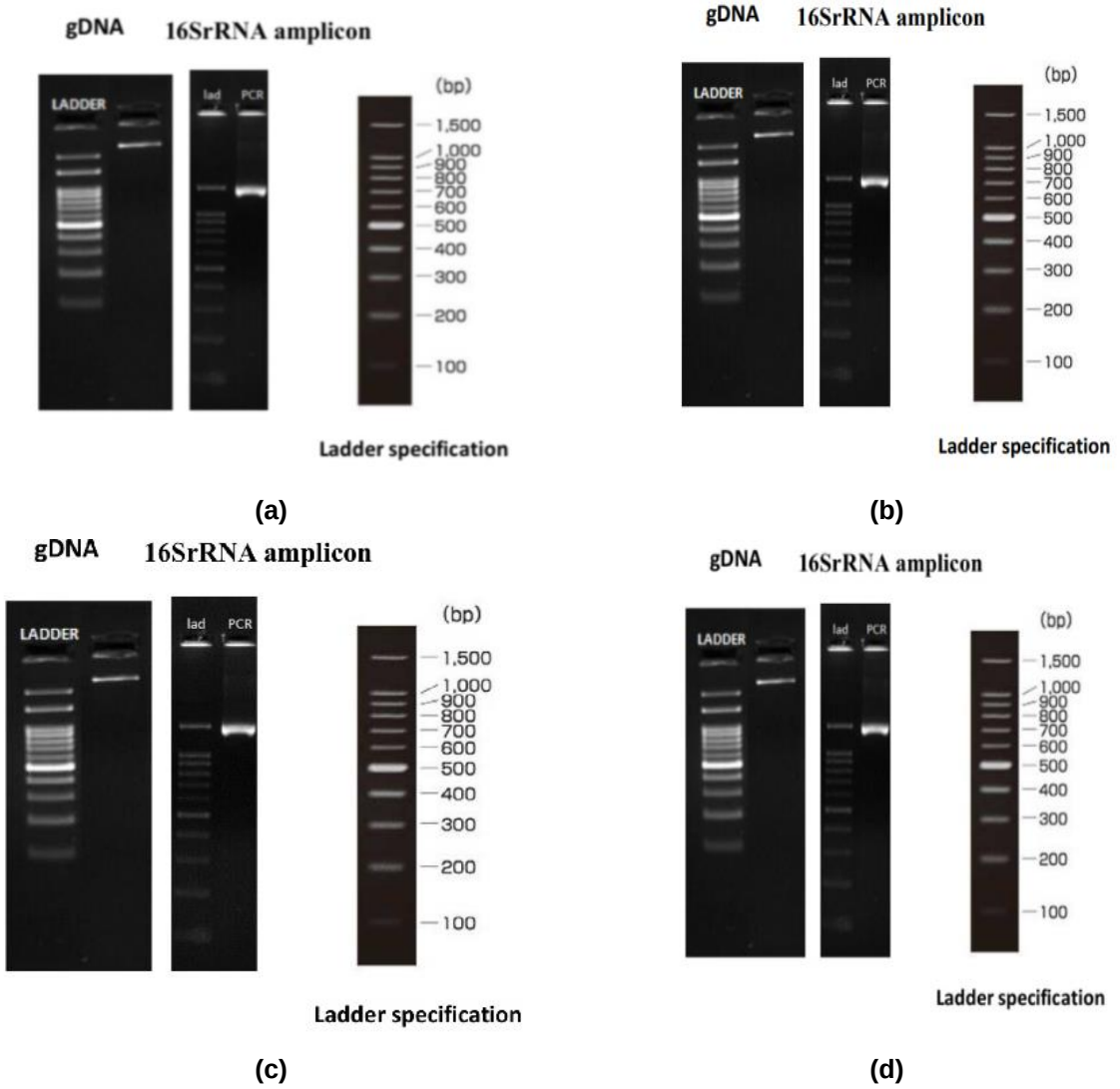

Fig 1. 16S rRNA gene (1500bp) amplification of bacterial strains. (a) PB-01KB *Pseudomonas mosselii*, (b) HR3-PM *Klebsiella aerogenes*, (c) PB-466 *Pseudomonas stutzeri* and (d) PB-424 *Lysinibacillus acetophenoni*

Table 3. BLAST result of PB01-KB

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Pseudomonas mosselii strain CFML 90-83 16S ribosomal RNA	2724	2724	100%	0	99.80%	1513	NR_024924.1
Pseudomonas entomophila L48 16S ribosomal RNA	2715	2715	100%	0	99.66%	1526	NR_102854.1
Pseudomonas plecoglossicida strain FPC951 16S ribosomal RNA	2699	2699	100%	0	99.46%	1498	NR_024662.1
Pseudomonas entomophila L48 16S ribosomal RNA	2695	2695	99%	0	99.46%	1515	NR_115336.1
Pseudomonas monteilii strain CIP 104883 16S ribosomal RNA	2686	2686	100%	0	99.33%	1517	NR_024910.1
Pseudomonas plecoglossicida strain NBRC 103162 16S ribosomal RNA	2662	2662	98%	0	99.52%	1462	NR_114226.1
Pseudomonas taiwanensis DSM 21245 strain BCRC 17751 16S ribosomal RNA	2660	2660	98%	0	99.52%	1469	NR_116172.1
Pseudomonas monteilii strain NBRC 103158 16S ribosomal RNA	2647	2647	98%	0	99.32%	1462	NR_114224.1
Pseudomonas monteilii strain CIP 104883 16S ribosomal RNA	2641	2641	99%	0	98.85%	1503	NR_112073.1
Pseudomonas parafulva NBRC 16636 = DSM 17004 16S ribosomal RNA	2641	2641	99%	0	98.79%	1484	NR_040859.1

Table 4. BLAST results of HR3-PM

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Klebsiella aerogenes KCTC 2190	2719	2719	1	0	0.9986	NR_102493.2
Klebsiella aerogenes strain NBRC 13534	2697	2697	99%	0	99.86%	NR_113614.1
Klebsiella aerogenes strain NCTC10006	2697	2697	99%	0	99.73%	NR_114737.1
Raoultella ornithinolytica strain JCM6096	2649	2649	99%	0	98.98%	NR_114736.1
Kluyvera cryocrescens strain NBRC 102467	2643	2643	99%	0	99.18%	NR_114108.1
Raoultella ornithinolytica strain CIP 103364	2641	2641	100%	0	98.92%	NR_044799.1
Raoultella planticola strain NBRC 14939	2636	2636	99%	0	99.04%	NR_113701.1
Yokenella regensburgei strain CIP 105435	2636	2636	100%	0	98.85%	NR_104934.1
Klebsiella aerogenes strain JCM 1235	2634	2634	97%	0	99.72%	NR_024643.1
Klebsiella pneumoniae strain DSM 30104	2625	2625	100%	0	98.71%	NR_117683.1

Table 5. BLAST results of PB-424

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Lysinibacillus manganicus DSM 26584 strain Mn1-7	2569	2569	99%	0	98.76%	NR_118533.1
Lysinibacillus acetophenoni strain JC23	2549	2549	98%	0	98.81%	NR_135864.1
Lysinibacillus massiliensis 4400831 = CIP108448 = CCUG 49529	2429	2429	96%	0	97.80%	NR_043092.1
Lysinibacillus chungkukjangi strain 2RL3-2	2412	2412	98%	0	97.00%	NR_109669.1
Lysinibacillus endophyticus strain C9	2401	2401	98%	0	96.75%	NR_146821.1
Lysinibacillus halotolerans strain LAM612	2350	2350	96%	0	96.94%	NR_134073.1
Lysinibacillus alkaliphilus strain OMN17	2344	2344	96%	0	96.67%	NR_136779.1
Ureibacillus defluvii strain DX-1	2342	2342	97%	0	96.30%	NR_133885.1
Lysinibacillus odysseyi 34hs-1 = NBRC100172 strain 34hs1	2335	2335	97%	0	96.28%	NR_025258.1
Lysinibacillus telephonicus strain S5H2222	2333	2333	96%	0	96.47%	NR_157637.1

Table 6. BLAST results of PB-466

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Pseudomonas stutzeri ATCC 17588 = LMG1119	2647	2647	100%	0	99.93%	NR_041715.1
Pseudomonas stutzeri strain CCUG 11256	2647	2647	100%	0	99.93%	NR_118798.1
Pseudomonas stutzeri strain NBRC 14165	2643	2643	100%	0	99.86%	NR_113652.1
Pseudomonas stutzeri ATCC 17588 = LMG11199	2641	2641	100%	0	99.86%	NR_103934.2
Pseudomonas stutzeri strain VKM B-975	2636	2636	100%	0	99.79%	NR_116489.1
Pseudomonas songnenensis strain NEAUST5-5	2556	2556	100%	0	98.82%	NR_148295.1
Pseudomonas chloritidismutans strain AW-1	2516	2516	100%	0	98.26%	NR_115115.1
Pseudomonas kunmingensis strain HL22-2	2497	2497	100%	0	98.05%	NR_133828.1
Pseudomonas guariconensis strain PCAVU11	2488	2488	100%	0	97.92%	NR_135703.1
Pseudomonas knackmussii B13	2486	2486	99%	0	98.04%	NR_117756.1

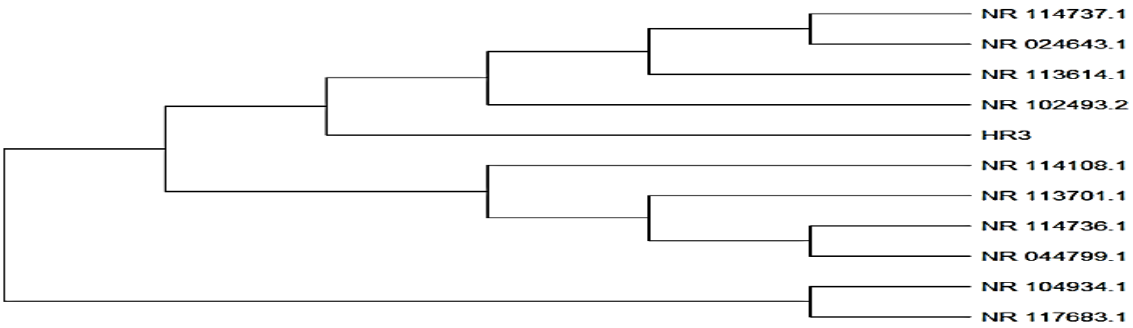

Fig. 2. Phylogenetic tree constructed by the neighbor-joining method derived from analysis of the 16S rRNA gene sequence of salt-tolerant PGPR bacterial isolate: HR3-PM

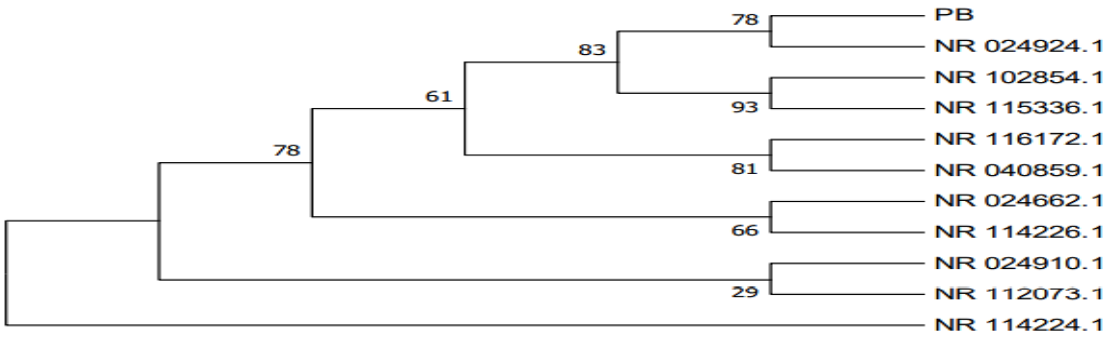

Fig. 3. Phylogenetic tree constructed by the neighbor-joining method derived from analysis of the 16S rRNA gene sequence of salt-tolerant PGPR bacterial isolate: PB01-KB

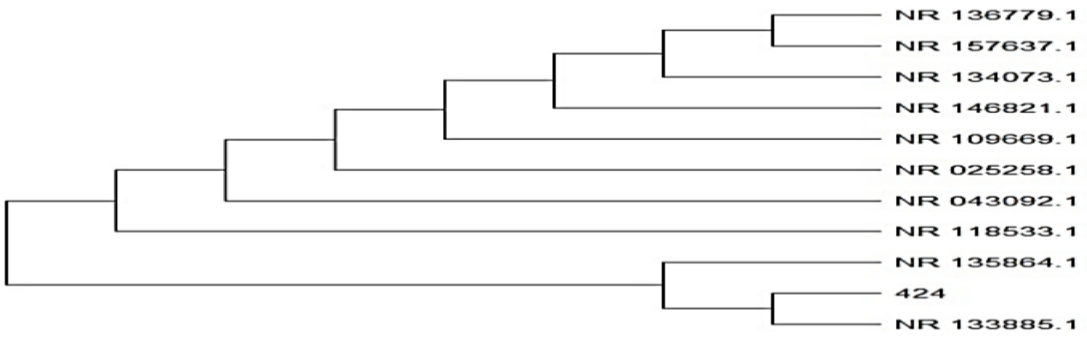

Fig. 4. Phylogenetic tree constructed by the neighbor-joining method derived from analysis of the 16S rRNA gene sequence of salt-tolerant PGPR bacterial isolate: PB-424

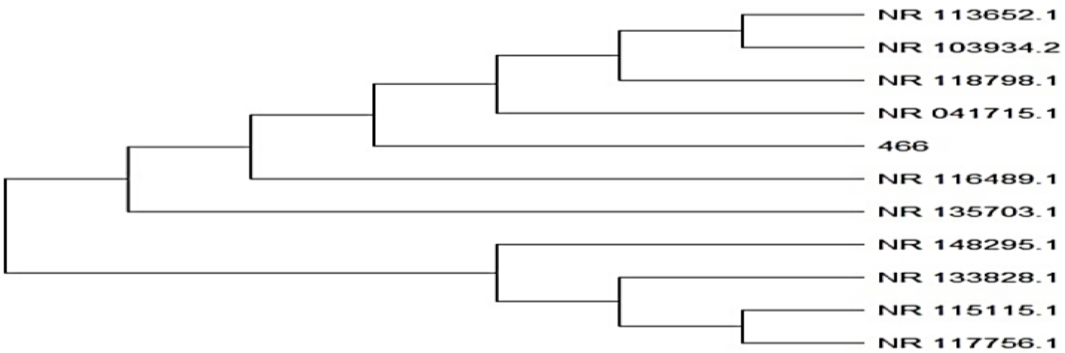

Fig. 5. Phylogenetic tree constructed by the neighbor-joining method derived from analysis of the 16S rRNA gene sequence of salt-tolerant PGPR bacterial isolate: PB-466

4. DISCUSSION

The increase in salt accumulation in soil is one of the most common environmental threats to plant yield and quality [45,46]. Salt-affected is reducing the cultivable area for agriculture by 1–2% every year, thereby reducing food production [47,48]. The characterization and molecular identification of halophilic bacterial strains (HR3-PM, PB01-KB, PB-424, and PB-466) isolated from salt-affected soil offer valuable insights into their adaptation mechanisms, potential agricultural applications, bioremediation potential, taxonomic classification, and evolutionary relationships. By understanding the morphological, biochemical, and physiological characteristics of these bacteria, including their ability to thrive in extreme environments and promote plant growth through traits like phosphate solubilization and indole-3-acetic acid production, we gain knowledge that can be applied to bioremediation efforts, sustainable plant growth practices, and environmental management strategies. Furthermore, molecular techniques such as PCR amplification and sequencing of the 16S rRNA gene enable precise taxonomic identification and phylogenetic analysis, elucidating the genetic diversity and evolutionary history of these halophilic bacteria. Damodarchari et al [49] reported similar results with Enterobacteriaceae spp. All four strains show positive results for IAA production and Phosphate solubilization. The salt-tolerant bacteria with PGPR activities may prove beneficial in managing salt-affected agricultural fields for crop improvement. Alternatively, halophilic bacteria and their genes can be mined for salt-tolerant PGPR activities or salt-tolerance traits can be transferred to crop plants, [50,51 and 52]. This holistic comprehension guides both scientific exploration and practical utilization of bacteria for beneficial purposes, enhancing understanding of salt-affected ecosystems.

5. CONCLUSION

In summary, halophilic bacteria have been isolated and characterized from salt-affected soils in Punjab and Haryana, India. These findings provide encouraging information on the possible uses of these bacteria in environmental management and soil alleviation. Indicative of their plant growth-promoting rhizobacteria (PGPR) activities, the four discovered strains, HR3-PM, PB01-KB, PB-424, and PB-466, display a variety of morphological, biochemical, and physiological characteristics, such as phosphate solubilization and indole-3-acetic acid (IAA) synthesis. Their close resemblance to well-known bacterial species including *Klebsiella aerogenes, Pseudomonas mosselii, Lysinibacillus acetophenoni,* and *Pseudomonas stutzeri* was shown by molecular research, which also validated their taxonomic categorization. Their evolutionary ties were further illuminated by phylogenetic research. These halophilic bacteria have the potential to tackle environmental stresses and support sustainable plant growth by reducing salt stress in soils. Their relevance in tackling global environmental concerns is further highlighted by their biotechnological potential, which includes applications in bioremediation and environmental management. To fully use the potential of these bacteria and incorporate them into workable strategies for managing salt-affected fields, additional study is necessary. This will help to ensure a more resilient and sustainable plant growth and environmental management future.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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