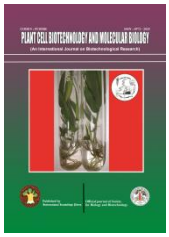

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Molecular Identification and Characterization of Indigenous Rhizobacteria from Haryana and Punjab and Their Inoculation Effect on *Prosopis cineraria*

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

India grapples with escalating soil salinity, projected to afflict 16.2 million hectares by 2050. Reforestation, vital in North India's sodic wastelands, counters severe degradation. Rhizobacteria that promote plant growth (PGPR) are essential for managing forests sustainably. These microbes help rhizosphere plants form symbiotic relationships that promote hormone production, nitrogen fixation, increased phosphate availability, iron sequestration, and defense against outside threats. The study aimed to isolate halo-tolerant microbial strains from salt-affected areas and studied their effect on tree species to alleviate their overall growth in Punjab and Haryana. We isolated 880 bacterial isolates from salt-affected areas in Punjab and Haryana; they have a variety of metabolic

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capabilities and are resistant to both salty and alkaline environments. Pot studies with a range of tree species verify the beneficial effects of particular bacterial strains, such as *Pseudomonas mosselli* and *Klebsiella aerogenes*, on soil &plant development metrics. Validation of these results by 16S rRNA gene sequencing highlights the potential of these isolates for soil remediation in salt-affected areas. The study highlighted the importance of microbial interventions in alleviating soil deterioration and fostering sustainable land use practices, and it suggests more field studies with different varieties of trees.

Keywords: PGPR; reforestation; sodic wastelands; soil salinity; 16S rRNA gene sequencing; sustainable land use practices.

1. INTRODUCTION

In the 21st century, global water scarcity, widespread pollution, and escalating soil salinization pose serious challenges. Prolonged soil mineral degradation and insufficient drainage contribute to salt accumulation, adversely impacting plant growth and yield. Around 6.727 million ha in India (2.1% of the geographical area is salt-affected, of which 2.956 million ha is saline and the rest 3.771 million ha is sodic [1, 2]. The losses are likely to increase manifold with the projected increase in salt-affected soils to 16.2 million ha by 2050 [3]. Climate change affects the water cycle, with rising temperatures impacting water availability and drying soils [4]. Agrochemical misuse harms soil biotic communities, contaminates the environment, and poses a public health concern [5, 6] while combustion of fossil fuels and emissions of greenhouse gases are accelerating global climate changes [7]. Soil salinity leads to nutrient deficiency in plants [8]. Soil salinity causes global crop losses and affects soil properties [9]. Land resources are the basis for human livelihood and societal development [10]. Since the 20th century, land degradation has escalated and aggravated due to ecological degradation, increased food demand of the growing population, rapid urbanization, industrialization, and indiscriminate use of land resources [11]. Deforestation (2.07 M ha) from 2001 to 2021 [12], intensive rainfall (>7.5 mm ha-1), uncontrolled grazing in 5.65 M ha [13], indiscriminate use (32 MT year-1) of fertilizers [14], and shifting cultivation in 7.6 M ha [15] are other major factors that aggravate the land degradation. According to SAC [16], at present, 97.8 M ha (29.7%) of the area is degraded in the country. Afforestation is not only a necessity for reducing pressure on natural forests but also a most desired land use, especially for reclaiming and rehabilitating degraded lands, particularly salt-affected soils. It is possible to increase wasteland productivity in terms of food, fuel wood, forage, medicinal drugs, and biodiversity if it is planted with trees. Salt-affected wastelands hold promise for agroforestry. Soil improvement in agroforestry is linked to biological nitrogen fixation, recycling of nutrients from deeper layers to the surface soil, building up soil organic matter (SOM) from above-ground and below-ground parts of plants, increasing soil microbial activity, improving soil enzyme activity, and enhancing the activity of arbuscular mycorrhizal fungi [17]. Soil physical properties play an important role in anchoring plants through root proliferation, whereas chemical and biological properties facilitate nutrient availability to the plants. The correlation between tree species and soil variables has been successfully demonstrated in tropical forests worldwide [18, 19]. Understanding these relationships helps in knowing how to best manipulate the ecosystem for better soil resilience [20]. Soils, along with competitive abilities and climate, play a leading role in determining species [21].

Bio-fertilizers, replacing inorganic ones, aid land rehabilitation, enhancing environmental quality by boosting biodiversity, carbon storage, water retention, and nutrient levels. [22-63]. Therefore, the application of beneficial microbiomes as biofertilizers in sustainable agriculture practices has emerged as an innovative and eco-friendly for improving soil fertility and plant growth [26-30]. Few native species have been established initially and subsequently, other species invaded and colonized forming a niche. Kushwaha et al, [31], reported that root-colonizing bacteria produce phytohormones that alleviated salinity-induced dormancy and elicited seedling growth. Moreover, [32] showed that *Pseudomonas* sp. and *Bacillus* promoted growth in stressed plants by producing indole acetic acid (IAA), siderophores, and solubilizing phosphates. ACC-deaminase-containing microorganisms inhibit ethylene synthesis, enhancing root growth [23, 34]. Lowered ethylene levels resulted in root growth and improved the survival of stressed

plants [35]. Microorganisms employ different strategies for stress tolerance [36]. 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 [37]. Some secondary metabolites such as flavonoids, phytoalexins, phenylpropanoids, and carotenoids have been documented in stressed plants inoculated with microorganisms [38, 39]. Thus, we aimed to isolate halo-tolerant microbial strains from salt-affected areas and studied their effect on tree species to alleviate their overall growth and sustainable land use practices [25].

2. MATERIALS AND METHODS

2.1 Study Areas

This study is centered on soil samples from districts in Kaithal and Fatehabad, Punjab, which are damaged by salinity. The predominant soil types of Kaithal, which spans 2317 km^2 are saline (6122 ha) and sodic (804 ha). Of the region Kaithal, about 11.3% (26301 ha) is impacted by salt; 6.8% (15,986 ha) of it is sodic, and 4.4% (10,315 ha) is saline. The district of Fatehabad, which spans 2520 km^2, is situated in the Indo-Gangetic basin, which has a tropical climate with hot summers and chilly winters. The district's subsurface water is typically brackish or saline, and irrigation is provided via the Bhakra and Western Yamuna canals. Covering 2630 km^2, the Muktsar district in the southwest of the state suffers from calcareous soils, nutrient deficits, salinity, alkalinity, and wind erosion. With three sub-divisions and seven blocks, Bathinda district is located in the southern region of Punjab, between 29°33' and 30°36' N latitude and 74°38' and 75°46' E longitude. The area, which is made up of sandy soil from the Indo-Gangetic alluvium, has sporadic dunes that lean eastward. The study provides an overview of the topographical and agricultural features of these areas, highlighting the difficulties caused by salt-affected soils.

2.2 Collection of Soil

A total of 605 soil samples were collected from Kaithal, Fatehabad in Haryana, and Muktsar, Bhatinda in Punjab. With the aid of a trowel, soil samples were taken at predefined depths of 0–30, 30–60, and 60–90 cm (Khurpi). A distinct plastic zipper bag with a label was used to pack the soil sample, which was then brought to the laboratory for examination. In the laboratory, samples were split into two sections: one for physicochemical and the other for microbiological analysis. The microbiological samples were kept in a deep freezer at 4^0 C.

2.3 Analysis of Soil Samples

2.3.1 Physicochemical analysis of soil

Soil pH was determined using a pH meter with 1:2.5 soil water ratios. Soil organic carbon (SOC) was determined after [40] (Walkley and Black Method, 1934). Soil texture was analyzed by the Hydrometer method [41] (Bouyoucous, 1962). Soil available nitrogen was analyzed by the method given by [42] (Subbiah and Asija (1956). Potassium by [43] (Hanway and Heidel, 1952), Determination of available phosphorus is by the Olsen method [44].

2.3.2 Bacteriological isolation

The serial dilution method given by Johnson and Curl 1972 [45] was followed using a Nutrient Agar medium by Sisco Research Laboratories Pvt. Ltd. (SRL). A total of 880 bacterial isolates of soil bacteria were isolated.

2.4 Statistical Analysis

Data were summarized as mean ± SD (standard deviation). Pearson correlation analysis was done to assess associations between the variables. A two-tailed values less than ($p<0.05$) were considered statistically significant. Analysis was performed using SPSS software (version 16.0).

2.5 Biochemical Test for Bacterial Isolates

Ammonia excretion (Cappucino & Sherman method) [46], Assay for IAA (Glickmann & Dessaux, 1995) [47], Phosphorus solubilization Edi- Premono [48], Siderophore productions Schwyn and Neilands [49], dextrose fermentation test by Hugh and Leifson [50], mannitol fermentation test, and lactose fermentation test [51, 52]

3. RESULTS AND DISCUSSION

3.1 Physicochemical Properties of Soil in Bhatinda and Muktsar of Punjab and Kaithal and Fatehabad of Haryana

The soil characteristics of Punjab's Bhatinda and Muktsar districts are primarily alkaline, with pH

values ranging from slightly to extremely alkaline throughout the pre-monsoon and post-monsoon seasons. Exchangeable salt, calcium, and magnesium are responsible for the alkalinity, which is further exacerbated by inadequate irrigation and drainage water quality—notably, leaching and rainfall-induced soil acidification cause a notable drop in soil pH during post-monsoon seasons. Electrical conductivity (EC) measurements revealed that Punjab and Haryana have different levels of soil salinity pre- and post-monsoon, which affects soil quality and fertility dynamics. The study also looked at organic carbon content and found that because organic matter decomposes quickly in semi-arid environments [53], levels are often low. The surface soil layer (0–30 cm) had a significant ($P<0.05$) higher content of SOC due to the presence of more organic content in the soil surface layer [54]. The analysis of critical nutrients revealed that available nitrogen was 'very low' to 'low,' with pre-monsoon levels greater due to organic carbon mineralization [55]. Available potassium and phosphorus levels in Bhatinda and Muktsar districts were classified as "low" to "medium," showing seasonal variations in mineral accumulation. The study underscored seasonal soil quality variations, with post-monsoon seasons generally indicating improved conditions. Principal component analysis highlighted the significance of soil organic carbon and electrical conductivity. Continuous intense farming was linked to land degradation and nutrient depletion. The findings provide crucial insights for sustainable soil management in Bhatinda, Muktsar, Kaithal, and Fatehabad districts.

3.2 Soil Texture Properties of Bhatinda and Muktsar of Punjab and Kaithal and Fatehabad of Haryana

The sand was the predominant proportion in all land uses, according to the distribution of soil fractions in the current study. In Punjab, the soil texture was typically sandy loam. The two most common textural classes in Haryana were sandy loam and loamy sand. Additionally, it has been observed that increased organic matter content was linked to higher silt and clay content because trash fell under Safeda forest (SF) land use [56, 57]. The majority of the soil type is made up of sand and silt, while very few places show any trace o f clay.

3.3 Soil Microbial Morphological and Biochemical Characterization Bacterial Isolates of Bhatinda and Muktsar of Punjab and Kaithal and Fatehabad of Haryana

Bacterial isolates from various locations were methodically gathered and rigorously characterized morphologically and biochemically. According to color and colony morphology, the study found that the maximum numbers of bacterial species were found between 0 and 30 cm below the surface. Out of the bacteria that were recovered from Punjab's salt-affected soils, 42% of them were Gram-positive and 58% were Gram-negative. These bacteria were both halophilic and halotolerant. Biochemical analyses of these isolates revealed that 40% of them produced indole acetic acid, 62% phosphate-solubilizing, 60% ammonia-producing, 50% fermented dextrose, 40% lactose, and 45% mannitol. Batista et al, [58] demonstrated that the genome of the *B. thuringiensis* strain RZ2MS9 harbors the complete set of genes required for indole acetic acid production. Based on Cultural and Biochemical characterization, the isolates were identified to be probably *Streptococcus spp, Bacillus spp, Enterobacter spp, Clostridium spp, Halobacterium spp, Serratia spp, Pseudomonas spp, Azospirillum spp, Rhizobium spp, Acinetobacter spp, Staphylococcus spp, Azotobacter spp, E.coli, Micrococcus spp, Serratia spp, Rhizobium spp, Proteus pp, Flavobacterium spp*, and *Klebsiella* spp. Similarly, in a comprehensive study of soil bacteria in the Kaithal and Fatehabad districts of Haryana, the bacterial isolates underwent meticulous morphological and biochemical characterization. In this context, 44.5% of the isolates were identified as Gram-positive, while 55.5% were Gram-negative. Biochemical tests revealed that 45% of the isolates were indole acetic acid-producing, 63.3% were phosphate-solubilizing, 63.3% were ammonia-producing, 70% were dextrose-fermenting, 40% were lactose-fermenting, and 43.87% were mannitol-fermenting. The isolates were identified through biochemical characterization and carbohydrate fermentation as *Enterobacter* spp, *Pseudomonas* spp, *Azospirillum*spp, *Klebsiella*spp, *Micrococcus* spp, *Serratia*spp, *Bacillus*spp, *Streptococcus* spp, *Pseudomonas* spp, *Staphylococcus* spp, *Acinetobacter* spp, *Rhizobium* spp, *Halobacterium* spp, *Flavobacterium* spp, *Clostridium* spp, *Proteus vulgaris*,

Azotobacter spp, *Halobacterium* spp, *Pseudomonasfluorescens*, *Mesorhizobium*spp, *Streptococcus*spp, *E. coli*, *Micrococcus*spp, *Halobacterium*spp, *Lactobacillus acidophilus*, *Klebsiella*spp, *Shigella*spp, *Rhizobium*spp, and *Azotobacter* spp. Pathak et al, [59] screened 39 *Bacillus* isolates for plant growth-promoting traits *in Vitro and* found that 48.7% of isolates were IAA producers, 38.4% of the isolates showed the ability to solubilize the phosphate, and 71.8% of isolates were able to produce ammonia. All the isolates showed the ability to produce hydrogen cyanide and protease. In another study, thirteen bacteria were isolated from salt-polluted soil [56], with HB6P2 and HB6J2 exhibiting the highest salt tolerance at 10%. All salt-tolerant isolates produced hydrogen cyanide, with HB6J2 showing the maximum production, while ammonia production was highest in HB6P2. Identified as *B.paramycoides*, *B.amyloliquefaciens*, and *B.pumilus* through 16S rDNA sequencing, these bacteria hold potential for nutrient recycling through phytostimulation and phytoremediation.

3.4 Molecular Identification of Salt-Tolerant Bacterial Isolates with PGPR Activities

The 16S rRNA gene sequence was used to carry out BLAST with the 'nr' database of the NCBI GenBank database. Based on the maximum identity score first ten sequences were selected and aligned using the multiple alignment software program Clustal W. Distance matrix and the phylogenetic tree constructed using MEGA 10.

3.5 PCR Amplification of 16S rRNA Gene

16S rRNA gene amplification was investigated in two distinct DNA strains (HR_319 and PB-446). As illustrated in Fig.1, it was discovered to have a single band of high-molecular-weight DNA on a 1.0 percent agarose gel. A portion of the 16SrRNA gene was amplified using 16SrRNA-F and 16SrRNA-R primers. There was only one unique 1500 bp PCR amplicon band found when the sample was resolved on an agarose gel. The PCR amplicon was purified to get rid of contaminants. Using the BDT v3.1 Cycle sequencing kit, forward and reverse DNA sequencing reactions of PCR amplicons were carried out on an ABI 3730xl Genetic Analyzer using 16SrRNA-F and 16SrRNA-R primers. A consensus sequence for the 16S rRNA gene was generated from forward and reverse sequencing data using aligner software.

3.6 Blast Result of HR_319 and PB-446

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model [61]. In HR_319, The tree with the highest log likelihood (-2462.79) is shown and in PB-446, the tree with the highest log likelihood (-2285.02) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1479 positions in the final dataset in HR_319 strain while there were a total of 1408 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [62].

The evolutionary tree of strain PB-446 showed a close resemblance with the *Pseudomonas mosselii* strain (99.80%), *P.entomophila* (99.66%), and *P.plecoglossicida* (99.46 %) with this strain. Hence it was designated as *Pseudomonas mosselii 9* (Table 1). The evolutionary tree of strain PB-446 showed a close resemblance with *P.mosselii* (Fig.2) On the other hand, strain HR_319 showed a close resemblance with *K.aerogenes*, *K.aerogenes*, and *K.cryocrescens.* Hence it was designated as*K.aerogenes* (Table.2). While the evolutionary tree of strain HR_319 showed its close resemblance with *K.aerogenes* (Fig. 2). The submission of 16S rRNA sequence to the NCBI GenBank and found the accession number of both sequences (PB-446 & HR_319) are MW475284 and MW479151 respectively. These two strains are halotolerant and possess adaptive mechanisms to tolerate hypersaline conditions.

In pot trials (Fig 5 & 6), the research explored the impact of diverse combinations of bacterial inoculums, chemical fertilizers, and compost on the growth of tree Prosopis cineraria. Isolated bacterial strains like *K.aerogenes* (MW479151) and *P.mosselii* (MW475284) were identified, originating from Ratiya and Surewala agriculture, respectively, in Punjab and Haryana. The study

investigated their effects on germination and growth, utilizing soil from the same region. The study focused on the inoculation of two bacterial strains, *K.aerogenes* (MW479151) and *P.mosselii* (MW475284), with various treatments involving seeds, composts, and NPK fertilizers applied individually or in combination. Treatments were meticulously designed: T2 involved seed inoculation only, T7 had seeds with NPK and inoculation, T12 had seeds treated with compost and inoculum, T17 included seeds treated with a full mix of compost, NPK, and inoculum, T22 featured seeds treated with a half mix of compost, half mix of NPK, and inoculum, T27 had seeds treated with half compost, full NPK, and Inoculum mix, and T32 involved seeds treated with a full mix of compost, half mix of NPK, and inoculum.

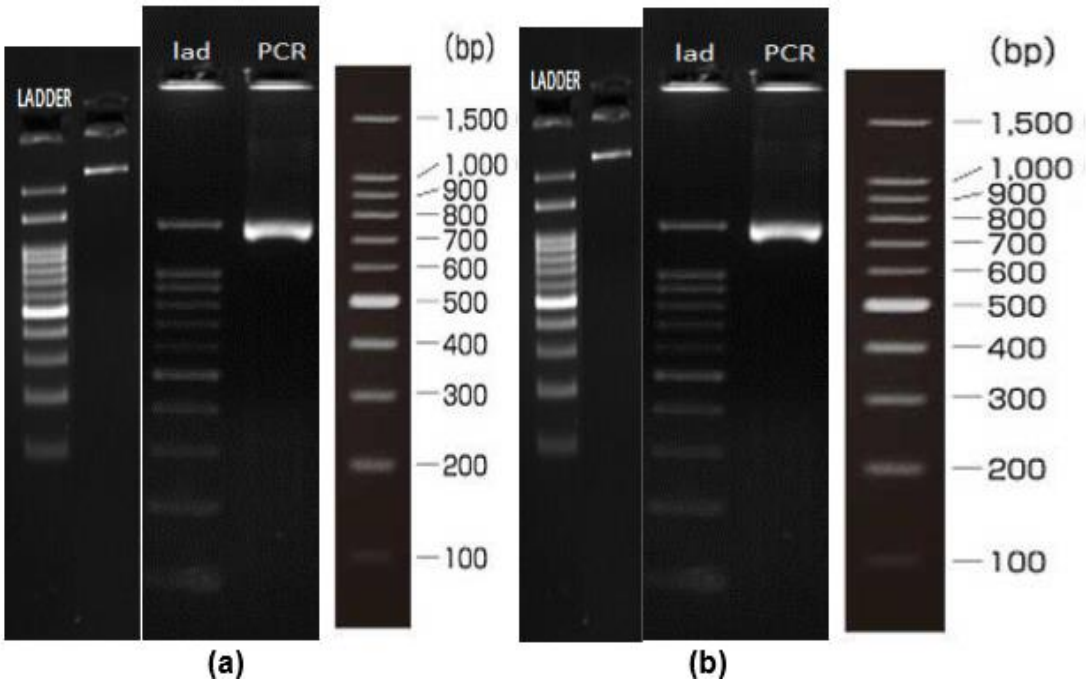

Fig. 1. 16SrRNA gene (1500bp) amplification of bacterial strain (a) HR_319 and (b) PB-446

Table 1. Blast result of PB-446

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

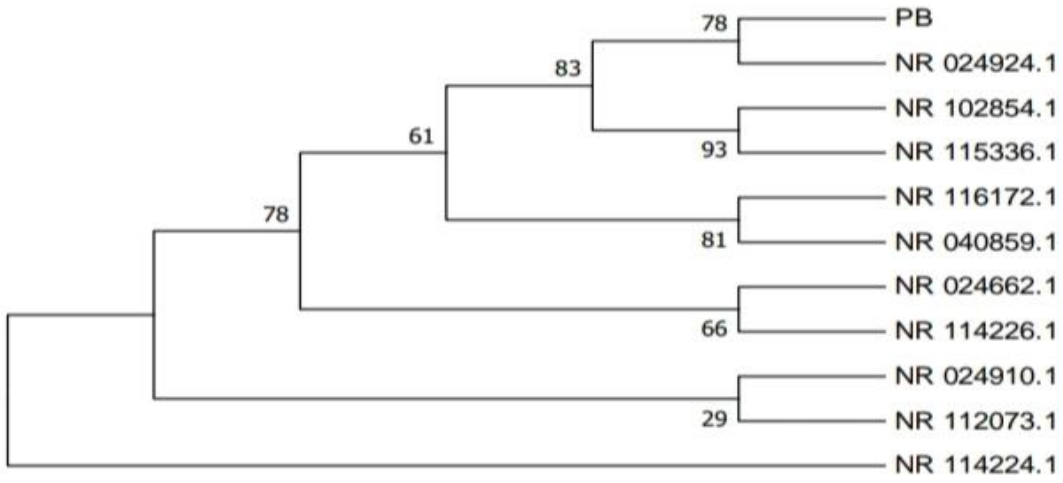

Fig. 2. Evolutionary relationship of taxa concerning PB-446

Table 2. Blast result of HR_319

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

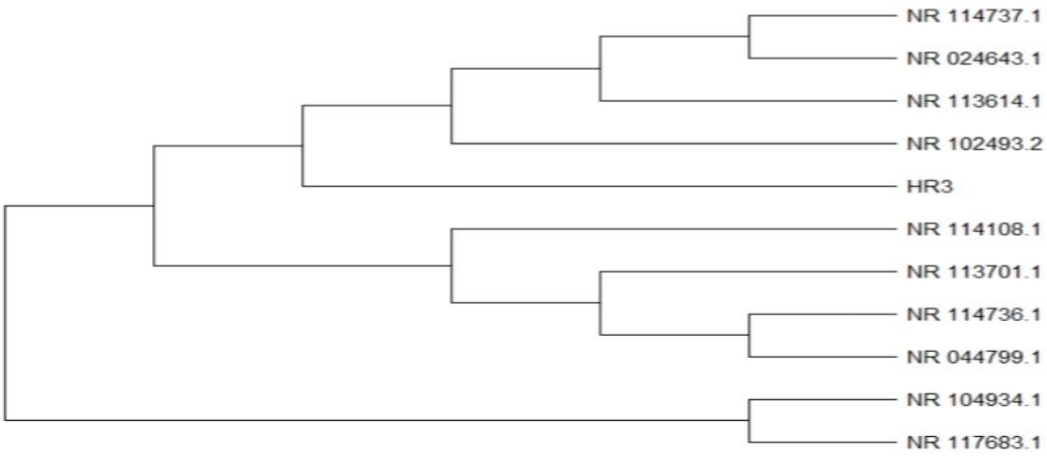

Fig. 3. Evolutionary relationship of taxa concerning HR_319

3.7 Study of the Effect of Bacterial Inoculation on *P. cineraria*in a Pot Experiment

Similar treatments were applied for *P.mosselii*, denoted as T5, T10, T15, T20, T25, T30, and T35. The growth parameters of specific plant species were assessed under these diverse treatments. The length of shoot and root was recorded on 30 DAS- days after sowing (Fig 4). In another study, Inoculation of *B. pumilus* strain JPVS11 improved the growth performance of rice as compared to non-inoculated and showed a significant ($P<0.05$) enhancement of plant height (12.90–26.48%), root length (9.55–23.09%), plant fresh weight (12.33–25.59%), and dry weight

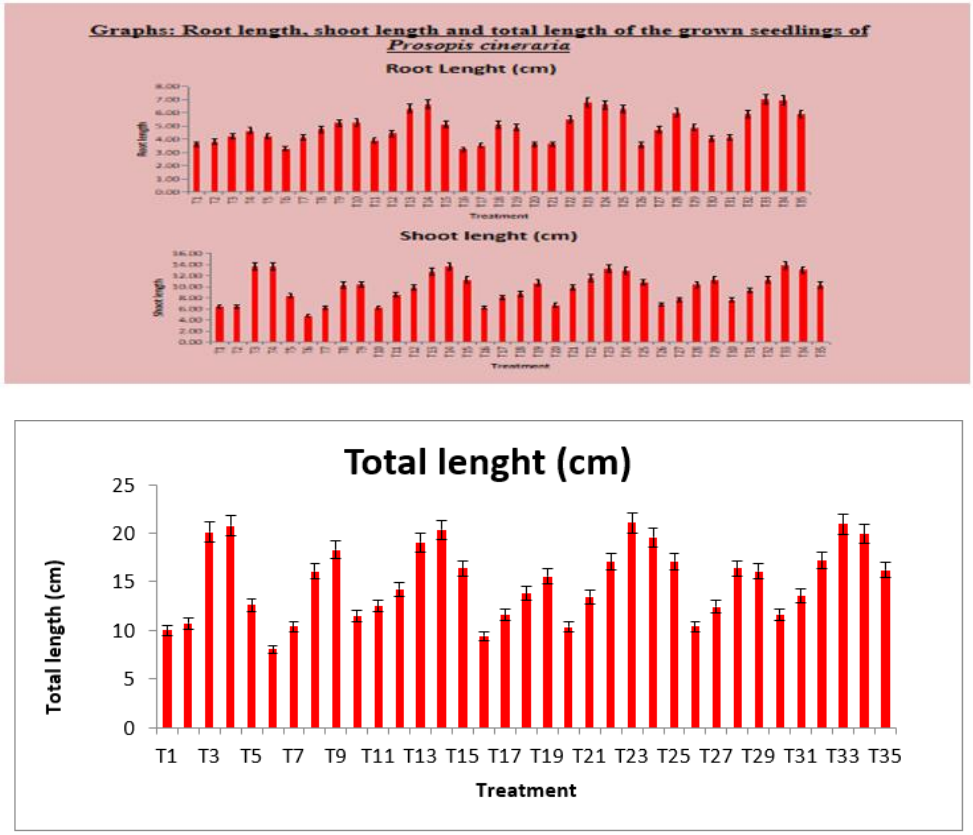

Fig. 4. Graphical representation of Root length, shoot length, and total length of *Prosopis cineraria*

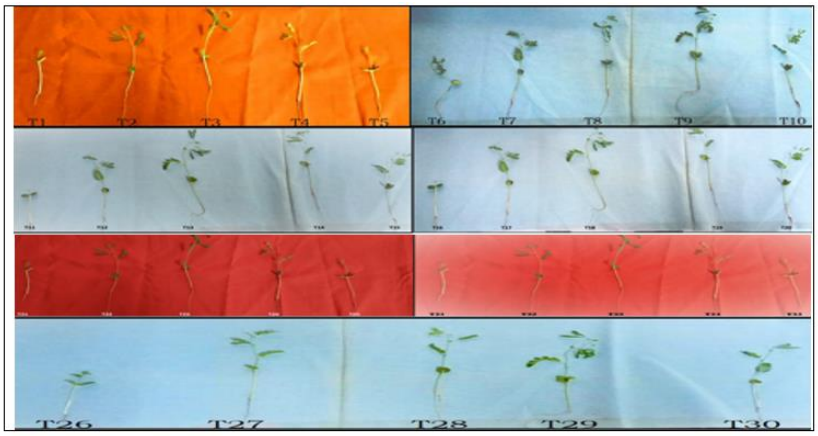

Fig. 5. Effect of different treatments on seedling growth of *Prosopis cineraria*

Fig. 6. Pot experiment results of *Klebsiella aerogenes* and *Pseudomonas mosselii* strain on *Prosopis cineraria*

(8.66–30.89%) were recorded [63].In another study, Two halotolerant bacterial isolates, GN-5 and JR-12, demonstrated robust plant growth promotion in Pisum sativum L. under varying saline conditions. JR-12 increased germination by 35.7%, shoot length by 46%, root length by 79.3%, and fresh weight by 86.84%. Similarly, GN-5 enhanced germination percentage by 42.8%, shoot length by 52.9%, root length by 79.3%, and fresh weight by 99.3% compared to the 200 mM control plants [64].

4. CONCLUSION

The study's conclusion emphasizes the vital impact that microbial interventions—in particular, PGPR—have in reducing soil salinity and encouraging sustainable land use practices in areas afflicted by salt, such as Punjab and Haryana. The work emphasizes the potential of halo-tolerant microbial strains—like *Pseudomonas mosselii* and Klebsiella aerogenes—for soil cleanup and improved plant growth through their isolation and characterization. These strains' tolerance for saline settings is further confirmed by their molecular identification using 16S rRNA gene sequencing. Pot experiments show that bacterial inoculation has a beneficial influence on the growth of species of trees such as Prosopis cineraria, indicating potential uses in reforestation initiatives. Overall, these results highlight the significance of using microbial biodiversity for sustainable agroforestry and soil rehabilitation, providing encouraging paths for tackling environmental issues and fostering ecosystem resilience. To strengthen and confirm these results and guarantee the successful application of microbial-based land restoration techniques, more field research involving a variety of tree species is advised.

COMPETING INTERESTS

The authors have declared that no competing interests exist.

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