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Original Research

Multifarious Plant Growth-Promoting Rhizobacterium *Enterobacter* sp. CM94-Mediated Systemic Tolerance and Growth Promotion of Chickpea (*Cicer arietinum* L.) under Salinity Stress

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Abstract

Background: Chickpea is one of the most important leguminous crops and its productivity is significantly affected by salinity stress. The use of ecofriendly, salt-tolerant, plant growth-promoting rhizobacteria (PGPR) as a bioinoculant can be very effective in mitigating salinity stress in crop plants. In the present study, we explored, characterized, and evaluated a potential PGPR isolate for improving chickpea growth under salt stress. **Methods**: A potential PGPR was isolated from rhizospheric soils of chickpea plants grown in the salt-affected area of eastern Uttar Pradesh, India. The isolate was screened for salt tolerance and characterized for its metabolic potential and different plant growth-promoting attributes. Further, the potential of the isolate to promote chickpea growth under different salt concentrations was determined by a greenhouse experiment. **Results**: A rhizobacteria isolate, CM94, which could tolerate a NaCl concentration of up to 8% was selected for this study. Based on the BIOLOG carbon source utilization, isolate CM94 was metabolically versatile and able to produce multiple plant growth-promoting attributes, such as indole acetic acid, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, siderophore, hydrogen cyanide (HCN), and ammonia as well as solubilized phosphate. A polyphasic approach involving the analysis of fatty acid methyl ester (FAME) and 16S rRNA gene sequencing confirmed the identity of the isolate as *Enterobacter* sp. The results of greenhouse experiments revealed that isolate CM94 inoculation significantly enhanced the shoot length, root length, and fresh and dry weight of chickpea plants, under variable salinity stress. In addition, inoculation improved the chlorophyll, proline, sugar, and protein content in the tissues of the plant, while lowering lipid peroxidation. Furthermore, isolate CM94 reduced oxidative stress by enhancing the enzymatic activities of superoxide dismutase, catalase, and peroxidase compared to in the respective uninoculated plants. **Conclusions**: Overall, the results suggested that using *Enterobacter* sp. CM94 could significantly mitigate salinity stress and enhance chickpea growth under saline conditions. Such studies will be helpful in identifying efficient microorganisms to alleviate salinity stress, which in turn will help, to devise ecofriendly microbial technologies.

Keywords: PGPR; chickpea; salt tolerance; lipid peroxidation; antioxidant enzymes

1. Introduction

Historically, chickpea plant (*Cicer arietinum* L.) has been one of the most significant component of the global cropping system. The total annual world production of chickpeas is 15.0 million tons, with India being the major chickpea-producing country, creating 73% of the global annual production [1]. Considering its high nutritional content, such as protein, energy, fiber, vitamins, and minerals, it is considered a cheaper substitute for meat [2]. Thus, it fills up the protein gap in the daily food intake of a growing Indian and global population. Moreover, legumes, such as chickpea, fix a considerable amount of nitrogen through biological nitrogen fixation, which has a significant contribution towards sustainable agriculture production [3]. However, due to their sessile nature, chickpea crop is considered susceptible to salt stress, and during the past few years, has faced serious global yield losses of 8.0–10.0% [4]. Globally, salt stress affects more than 800.0 million hectares (mha) of land and 20% of irrigated agricultural land [5]. Nearly 6.74 mha of land in India is under severe salinity stress [6]. Twelve states and one union territory in India covering 44% of the land are affected by soil salinity. Kaur *et al.* [7] reported that due to salinity, there has been a reduction of 36.1%–65.0% in chickpea yield at EC_{iw} 6 dS m^{-1}, which further increased to 81.0%–98.5% under EC_{iw} 9 dS m^{-1}. Plants under salt stress have to combat two serious impacts: (i) the ionic disequilibrium, due to an increase in the uptake of Na^+ or decrease in the uptake of Ca^{2+} and K^+ from the soil; and (ii) the osmotic imbalance, due to which the osmotic potential of the soil drastically

reduces [8]. Additionally, the high demand for energy and metabolic alterations due to the ionic disequilibrium and osmotic imbalance, can lead to a more lethal problem known as oxidative stress, which induces the generation of reactive oxygen species (ROS), such as hydroxyl radical ($OH^{\bullet}$), superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and singlet oxygen radical ($^1O_2$) [8]. The intracellular production of ROS induces oxidative injury to cell membranes by lipid peroxidation, photosynthetic pigments, lipids, and proteins [9]. Salt stress has adverse effects on vegetative and reproductive growth stages of the plants, which reduces the biomass and pod formation in chickpea plants [4]. Further, the salinity stress can also hinder soil microbial activities. Hence, effective salt mitigation strategies for this important nutritious crop are essential to sustain the increasing demand of food for the growing population [10].

To mitigate salt stress throughout the world, conventional breeding or genetic engineering approaches are developing salinity-tolerant plants and reclamation of saline soil by applying chemicals or organic amendments. However, the development of tolerant varieties requires a long time as well as a significant number of resources. Further, engineered plants should also comply with the regulations relating to genetically modified organisms. Therefore, to mitigate the negative effects of salt stress, researchers are now focusing on beneficial salt-tolerant plant growth-promoting rhizobacteria (PGPR) as a bioinoculant [11,12]. The PGPR performs a key role in plant growth through nitrogen fixation, augmenting nutrient (P, K, Zn, etc.) availability, the production of plant hormones (indole acetic acid and gibberellin) and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme, secretion of exopolysaccharides (EPS), antibiotics, and hydrolytic enzymes, to aid in coping with various stresses [8,13–15]. To date, many rhizobacterial genera, such as *Azotobacter*, *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Enterobacter*, *Streptomyces*, *Rhizobium*, *Exiguobacterium*, *Klebsiella*, *Serratia*, and *Ochrobactrum*, are well known for their roles in plant growth and production under salinity stress [16,17]. Among the different bacterial genera *Enterobacter*, which belong to the Enterobacteriaceae family, are adaptable and well-known root colonizers with multiple plant growth-promoting activities that have been found to help plants survive under different abiotic as well as biotic stress conditions [18,19]. Different *Enterobacter* spp., such as *E. cloacae*, *E. radicincitans*, *E. ludwigii*, *E. gergoviae*, and *E. asburiae* have been reported in different cropping systems, to promote the growth of economically important crops by rendering direct or indirect benefits [14,20–22]. In addition, some PGPR can induce the accumulation of osmolytes and modulate antioxidant enzymes to scavenge salinity-induced ROS and enhance plant growth [8]. Earlier studies have revealed that under salinity stress, salt-tolerant *Enterobacter* sp. P23 and *Enterobacter* sp. PR14 mitigated salinity stress in rice and millet seedlings by modulating defense-related antioxidant enzymes [21,23]. Recently, in another study, Ali *et al*. [24] reported a defensive role of rhizospheric bacteria *Enterobacter cloacae* PM23 in improving salt-stress tolerance and growth of maize. Thus, using such beneficial PGPR strains as bioinoculants, instead of synthetic chemicals, will not only increase plant growth and development but will also help to manage soil health under salt stress conditions.

Therefore, under this hypothesis, the present work was planned to explore and characterize salt-tolerant rhizobacteria from the chickpea rhizosphere and evaluate it for mitigation of salt stress in chickpea plants under greenhouse conditions.

2. Materials and Methods

2.1 Soil Sampling and Rhizobacteria Isolation

Chickpea (*Cicer arietinum* L.) plants grown in salt-affected fields of Mau district (25.9417° N latitude and 83.5611° E longitude) in Uttar Pradesh, India were uprooted with root-adhering soil. The rhizosphere soil sample was collected by gently shaking the plants. The soil was dried and sieved through a 2.0 mm mesh to remove clumps, roots, other plant material, etc., and shade-dried for two days on a polythene sheet. The electrical conductivity and pH of the sample were 6.6 dS m^{-1} and 9.0, respectively. The processed rhizospheric soil sample was serially diluted and spread on Nutrient agar (NA) (Cat. No. M001, HIMEDIA, Mumbai, MH, India) plates amended with 4% NaCl and incubated at 28 °C for 48 hours (h). After incubation, a fast-growing bacterial morphotype CM94 was selected and purified on a 4% NaCl-supplemented NA plate. The pure rhizobacterial isolate was preserved in 50% glycerol at –80 °C until further use.

2.2 Characterization of the Rhizobacteria

Morphological and biochemical characteristics of the CM94 isolate were determined by following Bergey's Manual of Determinative Bacteriology. Additionally, the culture was examined for colonial characteristics and subjected to microscopic analysis. Biochemical characterization was carried out using a Hi25™ Enterobacteriaceae Identification kit (Cat. No. KB003, HIMEDIA, Mumbai, MH, India), following the manufacturer's instructions. Production of extracellular hydrolytic enzymes, such as amylase, cellulase, protease, lipase, gelatinase, and urease was examined by adopting the protocol by Smibert and Krieg [25].

2.3 Screening of Isolate for NaCl Tolerance

The intrinsic tolerance of the CM94 isolate against different concentrations of NaCl was evaluated by inoculating the bacterial culture (10^8 cfu mL^{-1}) in 50 mL of nutrient broth (NB) containing NaCl ranging from 0 to 10% (w/v) at pH 7.0 and incubated at 28 ± 2 °C for 96 h, in triplicate. Growth, in terms of protein concentration, was monitored in 12 h intervals. The uninoculated nutrient broth was used as a blank.

2.4 Intrinsic Antibiotic Resistance

The sensitivity to different antibiotics *viz.* ampicillin (10 mcg), chloramphenicol (30 mcg), azithromycin (15 mcg), gentamicin (30 mcg), kanamycin (30 mg), erythromycin (15 mcg), penicillin (10 mcg), polymyxin-b (50 u), rifampicin (5 mcg), tylosin (15 mcg), streptomycin (10 mcg), vancomycin (30 mcg), nadifloxacin (10 mcg), and tetracycline (10 mcg) was determined by placing different antibiotic discs on the bacterial lawn growing on NA. The plates were incubated at 28 ± 2 °C for 48 h. After the incubation, the antibiotic resistance (–) and susceptibility (+) were recorded based on the presence and absence of a clear zone around each antibiotic disc.

2.5 Characterization of Plant Growth-Promoting Traits

Plant growth-promoting traits were evaluated under normal (no NaCl) and saline (2 to 8% NaCl) conditions. NaCl treatment was supplemented in the respective growth medium.

2.5.1 Phytohormone Indole Acetic Acid (IAA) Production

IAA production was estimated following the protocol of Bric *et al*. [26]. In brief, rhizobacterial cultures grown overnight were centrifuged (10,000 g for 5 min) and 2 mL bacterial supernatant, 4 mL Salkowski reagent (50 mL of 35% perchloric acid + 1 mL of a 0.5 M $FeCl_3$ solution), and orthophosphoric acid (~10 µL) were added and incubated for 30 min at room temperature. The quantity of IAA was measured at an absorbance of 530 nm. The quantity of IAA in the culture was extrapolated from the standard curve of pure IAA (0 to 100 µg mL^{-1}).

2.5.2 Determination of ACC Deaminase

ACC deaminase activity was determined as described by Penrose and Glick [27]. The pure rhizobacterial culture was spot inoculated on Dworkin and Foster (DF) salt minimal medium [28], amended with 0.10 M of ammonium sulfate or 3.0 mM of ACC for 48 h, and bacterial isolate growth was assumed as positive for ACC deaminase production. Quantitative activity of ACC deaminase at different NaCl concentrations was assayed by harvesting the bacterial cells through centrifugation (10,000 rpm for 10 min), followed by washing three times with Tris–HCl (0.1 M, pH 7.5), re-suspended in 1 mL of Tris–HCl (0.1 M, pH 8.5). Toluene (30 µL) was mixed into the cell suspension and homogenized by vortexing. An aliquot of toluene labialized cells was used for the ACC deaminase protein and activity assays [27].

2.5.3 Determination of P-Solubilization

The phosphate solubilizing ability of CM94 was determined by spot inoculation of the isolate on the National Botanical Research Institute's Phosphate Medium agar plate (NBRIP) [29] containing bromophenol blue and incubated for 48–72 h at 28 ± 2 °C. Observation of the color change around the bacterial colony on the NBRIP agar plate was considered a positive result for the phosphate solubilization ability. The phosphate solubilization index (PSI) was determined by measuring the halo zone diameter and colony diameter using the following formula:

PSI = (colony diameter + halo zone diameter) / colony diameter

2.5.4 Determination of Siderophore Production

The ability to produce siderophores by the CM94 isolate was tested, as per a previously described method by Schwyn and Neilands [30], using a chrome azurol S (CAS) agar plate. The appearance of a yellow to orange colored zone surrounding the bacterial colony was considered a positive result for siderophore production. Further the quantitative estimation of siderophore production was determined through the CAS-shuttle assay in MM9 medium.

2.5.5 Determination of Ammonia Production

An actively grown CM94 culture was inoculated into sterile peptone water (10 mL), containing different NaCl concentrations in separate tubes, and incubated for 48 h at 28 ± 2 °C. After incubation, 500 µL of Nessler's reagent was mixed with bacterial suspension in peptone water. The development of a dark yellow color indicated ammonia production [31].

2.5.6 Determination of Hydrogen Cyanide (HCN) Production

Production of hydrogen cyanide by the CM94 isolate was tested by adopting the standard method by Lorck [32]. Briefly, the isolate was streaked onto a NA plate supplemented with different salt concentrations and 4.4 g glycine/L. A strip of Whatman filter paper presoaked in Na_2CO_3 (2%) and picric acid (0.5%) solution, was kept in the lid of the Petri plate. Plates were sealed and kept at 28 ± 2 °C for 48–72 h. After incubation, the appearance of a red to orange color in the filter paper indicated HCN production.

2.6 Exopolysaccharide (EPS) Production

EPS production was estimated using the method of Qureshi and Sabri [33]. Here, 100 mL rhizobacterial culture (48 h), grown in NB medium with different NaCl concentrations, was centrifuged at 10,000 g for 15 min at 4 °C. EPS was precipitated from the supernatant with the help of 3 volumes of prechilled acetone. After 2 days, EPS (precipitated) was filtered using Whatman filter paper. The wet EPS on the filter paper was dried overnight at 58 °C and weighed again. The increase in the weight of the filter paper with EPS biomass indicated the positive result of EPS production.

2.7 Metabolic Characterization

Based on the carbon (95 C-substrates) utilization pattern, the metabolic characterization of the selected rhi-

zobacterial CM94 isolate was carried out using BIOLOG GNII (MicroPlate™ Biolog, Inc., Hayward, CA, USA), according to the manufacturer's instructions.

2.8 Fatty Acid Profiling

To analyze the fatty acid profile, fatty acids were extracted from the isolate, following the methods described by Sasser [34], and analyzed by gas chromatography (Agilent GC 7820A) using MIDI Sherlock software (Sherlock TSBA Library version 3.9, MIDI Inc., Newark, DE, USA) for fatty acid methyl ester analysis (FAME).

2.9 Molecular Identification through 16S rRNA Gene Sequencing

Genomic DNA from the overnight grown pure culture of CM94 was extracted following the method described by Pospiech and Neumann [35]. Amplification of the 16S rRNA gene was conducted using the universal forward primer PA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer PH (5′-AAG GAG GTG ATC CAG CCG CA-3′), following the PCR recipe and conditions described earlier [36]. The amplified products were purified using the Promega Wizard® SV Gel and PCR Clean-Up System (Cat. No. A9281, Promega, Madison, WI, USA) and sequenced by the Applied Biosystems ABI prism automated DNA sequencer (3130xl) at ICAR-NBAIM, Mau Nath Bhanjan (U.P.), India. The 16S rRNA gene sequence was searched against the GenBank database using the BLASTn tool, available on the NCBI platform, to identify the bacterial isolate. Further, a neighbor joining (NJ) phylogenetic tree was constructed using MEGA-X software, with Jukes–Cantor coefficient evolutionary distances [37,38].

2.10 Effect of Rhizobacterial Isolate CM94 on Chickpea Growth Promotion under Salinity Stress

To evaluate the growth promotion and salt-stress mitigation efficiency of the CM94 isolate on chickpeas, an experiment was conducted using 0, 75, and 150 mM NaCl concentrations in the greenhouse at ICAR-NBAIM, Mau Nath Bhanjan (U.P.). Briefly, the pure bacterial CM94 culture was grown overnight and harvested, centrifuged, washed, and resuspended in phosphate buffer (0.1 M, pH 7.0), to obtain a ~10^8 cfu mL^{-1} cell density. Chickpea seeds (*Cicer arietinum* L. cv Avrodhi) procured from ICAR-Indian Institute of Seed Sciences (IISS), Mau Nath Bhanjan (U.P.), India, were surface-sterilized with 70% ethanol for 3 min and 3% sodium hypochlorite for 3 min, followed by 5–6 wash with sterile distilled water (D/W). Then, the surface-sterilized seeds were shade-dried and suspended in bacterial suspension (1.5 m/10 g seeds) for 30 min at 120 g on a rotary shaker. Afterward, the seeds were air-dried for 1 h under aseptic conditions and sown (20 seeds pot^{-1}) in plastic pot boxes containing 2.0 kg of sterile river sand. The pot was treated or moistened with 0, 75, and 150 mM NaCl solutions with 60% of moisture holding capacity (MHC). The experiment was conducted in triplicate with a completely randomized block design. The whole set of experiments was independently repeated three times.

2.10.1 Plant Biometrics Analysis

Fifteen days after germination, chickpea seedlings from each treatment, were carefully uprooted and washed, and biometric parameters, such as root/shoot length, and fresh and dry weight were recorded. The dry weights of the shoot and root were recorded after drying at 70 °C for 24 h.

2.10.2 Biochemical Assays

Determination of Chlorophyll, Proline, Sugar, and Protein Content

Chlorophyll content was spectrophotometrically assayed by adopting the process of Arnon [39]. In brief, 100 mg of leaves were completely homogenized in 80% acetone and centrifuged at 5000 g for 5 min. Then, the supernatant was measured at 645 and 663 nm, and total chlorophyll content was calculated and expressed in mg g^{-1} FW.

Proline content was determined according to the protocol in Bates *et al.* [40]. Proline contents were spectrophotometrically measured by reading the absorbance of the ninhydrin reaction at 520 nm and the quantity of proline was examined using the standard curve of L-proline and expressed in µmol g^{-1} FW.

The total soluble sugars (TSS) of the rhizobacterial-treated and non-treated chickpea plants exposed to NaCl stress were estimated using the phenol sulfuric acid method [41]. The optical density of the cooled reaction mixture was measured at 620 nm, and the amount of total sugar (µg g^{-1} FW) was determined by the glucose standard curve.

The protein concentration was determined using the Bradford assay [42]. Spectrophotometrically the protein content was assayed at 595 nm and the protein concentration was determined using the BSA standard curve (bovine serum albumin).

2.11 Malondialdehyde (MDA) Content Estimation

The malondialdehyde content was estimated by adopting the procedure in Kumari *et al.* [43]. A total of 500 mg of leaves were ground in 5% (w/v) trichloroacetic acid and centrifuged at 12,000 g for 10 min. Then, 0.5% (w/v) of thiobarbituric acid (TBA) was mixed in 2.0 mL of diluted supernatant extract. Next, the reaction mixture was incubated at boiling temperature for 30 min and immediately terminated by transferring the reaction mixture to ice. Then, the reaction mixture was centrifuged at 12,000 rpm for 10 min, and measured spectrophotometrically at 532 nm. The MDA content in the samples was calculated using the MDA standard curve and expressed as nmol g^{-1} FW.

2.12 Antioxidative Enzyme Activity Assay

The crude extract for antioxidant enzymes present in the plant tissue was prepared by following the procedure

in Kumari *et al.* [43]. Briefly, a 1.0 g leaf sample was ground with potassium phosphate buffer (0.1 M, pH 7.4) containing 1 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), and 2% polyvinylpyrrolidone (PVP). The homogenate was centrifuged (10,000 g for 15 min at 4 °C) to collect the enzyme extract, which was further used in the antioxidant enzyme analysis.

2.12.1 Superoxide Dismutase (SOD) Activity

SOD activity was assayed spectrophotometrically, following the method of Beyer and Fridovich [44]. The reduction in nitro blue tetrazolium (NBT) was determined by recording the change in OD at 560 nm. Dark blanks were used to determine the enzyme units (U). For the photoreduction of 50% NBT, one unit of SOD was required. The activity of SOD was represented in U mg^{-1} protein.

2.12.2 Catalase (CAT) Activity

The CAT activity was determined spectrophotometrically by monitoring the disappearance rate of H_2O_2 at 240 nm [45]. The 2.5 mL reaction mixture was contained: potassium phosphate buffer (100 mM, pH 7), EDTA (0.1 mM), hydrogen peroxide (20 mM), and crude enzyme extract (50 µL). CAT activity was assayed by decrease in OD at 240 nm.. The catalase activity was expressed as a breakdown of 1 µmol of H_2O_2 min^{-1}.

2.12.3 Peroxidase (POD) Activity

POD activity was assayed spectrophotometrically using guaiacol as the substrate and recording the increase in OD at 436 nm at 30 °C [46]. The reaction was started by mixing 50 µL leaf extract with 3.0 mL of a solution consisting of guaiacol (1%), H_2O_2 (3%), and sodium phosphate buffer (100 mM, pH 6.0). After incubating in a water bath at 30 °C, the OD was measured at 436 nm, every 30 s for 5 min. The POD activity was expressed as U mg^{-1} protein.

2.13 Root Colonization Assay

Plants from every treatment were uprooted carefully and washed 3–4 times with sterilized distilled water and surface-sterilized, as described earlier. Afterward, the root samples were homogenized in sterile phosphate buffer, diluted, and spread (100 µL) on NA medium plates supplemented with a combination of rifampicin, penicillin, kanamycin, and tetracycline (selected based on initial intrinsic antibiotic resistance assay), using a concentration of 10 µg/mL for each. The plates were incubated for 24 h at 28 ± 2 °C. After the incubation, the obtained bacterial colonies were subcultured onto the same antibiotic-containing medium. To confirm the identity of the obtained bacterial culture, the genomic DNA of the culture was extracted and subjected to ERIC PCR along with the wildtype CM94 isolate. Enterobacterial repetitive intergenic consensus (ERIC) PCR was performed using a set of ERIC 1R (5′-ATGTAAGCTCCTGGGGATTCA-3′), ERIC 2 F (5′-AAGTAAGTGACTGGGGTGAGC-3′) primers. A 50 µL PCR reaction was carried out in a Thermal Cycler (G Storm GS4, Somerset, UK), under the following PCR conditions: 94 °C for 5 min (initial denaturation), 35 cycles at 94 °C for 1 min, at 50 °C for 1.5 min (annealing), and 65 °C for 8 min, and a final extension step at 65 °C for 10 min. Amplification was analyzed by electrophoresis using a 2.0% agarose gel containing ethidium bromide (0.5 µg mL^{-1}). To confirm the identity of the isolates, the ERIC PCR band patterns were compared by visualizing and documenting the gel in the Bio-Rad gel documentation system.

2.14 Statistical Analysis

All data were produced in triplicate and analyzed by analysis of variance (ANOVA) using Duncan's multiple range tests (DMRT) at the $p = 0.05$ level with SPSS (ver. 16.0, IBM SPSS Statistics, Chicago, IL, USA). The graphs were prepared using Microsoft Excel version 2019.

3. Results

3.1 Isolation and Basic Characterization of the Rhizobacteria

Based on the luxuriant and dominant growth on the NA medium amended with 4% NaCl (as a selective additive for isolation of salt-tolerant rhizobacteria), a CM94 rhizobacterial isolate was obtained from the rhizospheric soil of chickpea plants. Primary characterization of the isolate was performed through morphological and biochemical characteristics (Table 1). The results showed that the selected rhizobacterial isolate CM94 had circular, smooth, convex, cream-colored colonies with entire margins on the NA plates. Microscopic observation followed by Gram staining revealed that it was a Gram-negative rod-shaped bacterium. Further, CM94 was found to be positive for oxidase, catalase, citrate utilization, indole production, nitrate reduction methyl red, and ornithine utilization, while it was negative in the ONPG test, lysine utilization, Voges–Proskauer test, arginine, H_2S production, and phenylalanine deaminase. In addition, CM94 could produce hydrolytic enzymes, such as cellulase, protease, gelatinase, and urease (Table 1). An antibiotic sensitivity assay of CM94 revealed that it was resistant to penicillin, polymyxin, nadifloxacin, rifampicin, and tetracycline, whereas it was sensitive to ampicillin, azithromycin, chloramphenicol, erythromycin, kanamycin, streptomycin, tylosin, vancomycin, and gentamicin (**Supplementary Table 1**).

3.2 Screening for NaCl Tolerance

Results of intrinsic NaCl tolerance revealed that CM94 could grow in various salinities, ranging from 0 to 8% NaCl (w/v), with optimum growth in terms of cellular protein concentration was recorded at 2% NaCl (Fig. 1). At higher NaCl concentrations, bacterial growth was sluggish over the first 24 h. However, after a few hours and up to 72

Table 1. Morphological and biochemical characteristics of rhizobacteria CM94.

Characteristic	*Enterobacter* sp.CM94
Shape	Rod
Motility	+
Color	Cream
Gram reaction	–
NaCl tolerance (%)	up to 8%
pH tolerance (optimum 7.0)	6.0–9.0
Temperature tolerance	45 °C
Catalase activity	+
Oxidase activity	+
Citrate utilization	+
ONPG	–
Indole production	+
Methyl red test	+
VP test	–
Arginine	–
Nitrate reduction	+
Ornithine utilization	+
H_2S production	–
Phenylalanine deamination	–
Lysine utilization	–
Industrial enzymes	
Protease	+
Lipase	–
Amylase	–
Gelatin hydrolysis	+
Cellulase production	+
Urease production	+

(+) positive for test; (–) negative for test.
ONPG, O-Nitrophenyl-β-D-galactopyranoside; VP, Voges–Proskauer.

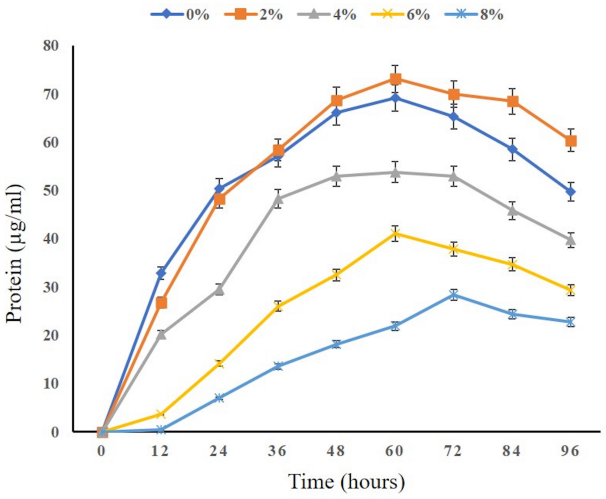

Fig. 1. Salt tolerance ability of the rhizobacterial isolate CM94 indicating cellular protein concentration under different salt concentrations at various durations. The highest protein concentration was recorded after 60 hours at a salt concentration of 2%. Each value in the graph indicates the mean of three replicates. Error bars represent mean ± standard error.

h of growth, it grew well in a saline medium before entering the stationary phase. In addition to the salinity, the CM94 strain could also grow in the liquid medium at a temperature range of 15–45 °C and a pH range of 4.5–9.0.

3.3 Determination of Plant Growth-Promoting Attributes and EPS Production

Different plant growth-promoting traits, such as IAA production, ACC deaminase, siderophore, ammonia, HCN, and P-solubilization by the CM94 isolate were evaluated *in vitro* at various salt (NaCl) concentrations (Table 2). The results showed that CM94 could produce 144.0 µg/mL IAA when grown without NaCl, whereas as the salt concentrations increased to 2, 4, and 6% NaCl, the IAA production decreased by 18.1, 44.6, and 56.3%, respectively. However, CM94 was unable to produce IAA at a NaCl concentration of 8% (Table 2). The ACC deaminase activity by CM94 was recorded as being 89.7 α-ketobutyrate nmol mg^{-1} h^{-1}. Yet, the activity declined by 12.6, 25.9, 53.7, and 84.8% as the salinity levels increased to 2, 4, 6, and 8% NaCl, respectively (Table 2). A similar observation was made for the siderophore production, where the isolate CM94 produced 69.1% unit siderophore when grown without salt, yet it decreased by 60.7% at 8% NaCl (Table 2). The P-solubilizing ability of the isolate CM94 was noticeable on the NBRIP medium plates, where it could change the color around the colony. Here, the CM94 isolate showed the highest (4.1) phosphate solubilization index (PSI) without any NaCl, while the solubilization ability decreased by 17.8, 42.2, 56.1, and 86.9% at 2, 4, 6, and 8% NaCl, respectively (Table 2). Furthermore, CM94 was able to produce ammonia and HCN. However, as the NaCl concentration increased, these activities were also found to decrease (Table 2).

The CM94 EPS production was also determined. The CM94 isolate produced 1.97 g EPS/100 mL with no salt stress (0% NaCl). Interestingly, it was observed that CM94 could produce EPS at all NaCl concentrations, with the highest (2.82 g/100 mL) at 4% NaCl (Table 2). Overall, the results of the PGP attributes and EPS production showed that the isolate could retain its PGP activities even at higher salt stress conditions.

3.4 Carbon Utilization and Fatty Acid Profile

The metabolic potential of the CM94 isolate, based on the utilization of different C-sources, was determined using the BIOLOG (R) system. The results showed that CM94 was metabolically active and among the ninety-five different C-sources, isolate CM94 utilized 64 C-sources, including 2 amines, 15 different amino acids, 22 carbohydrates, 18 carboxylic acids, 5 polymers, and 2 miscellaneous carbon sources (**Supplementary Table 2**).

Table 2. Plant growth-promoting attributes of CM94 isolate.

NaCl concentration	IAA (µg/mL)	ACC deaminase (α-ketobutyrate nmol mg^{-1} protein)	P-solubilization index (PSI)	Siderophore (% unit)	Ammonia production	HCN production	EPS production (g/100 mL)
0%	146.04 ± 3.35	89.71 ± 3.23	4.1 ± 0.14	69.16 ± 2.91	+++	+++	1.97 ± 0.17
2%	119.51 ± 2.62	78.35 ± 1.82	3.37 ± 0.12	65.72 ± 2.48	+++	+++	2.53 ± 0.12
4%	80.89 ± 4.48	66.42 ± 1.64	2.36 ± 0.14	58.57 ± 2.6	+++	++	2.82 ± 0.12
6%	63.7 ± 2.48	41.5 ± 1.65	1.80 ± 0.09	45.52 ± 2.87	++	++	1.63 ± 0.12
8%	-ND-	13.56 ± 1.21	0.53 ± 0.05	27.14 ± 0.87	+	-	0.76 ± 0.05

'+++': high production; '++': medium production; '+': low production; '-ND-': not detected.

IAA, indole acetic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; HCN, h ydrogen cyanide; EPS, exopolysaccharides.

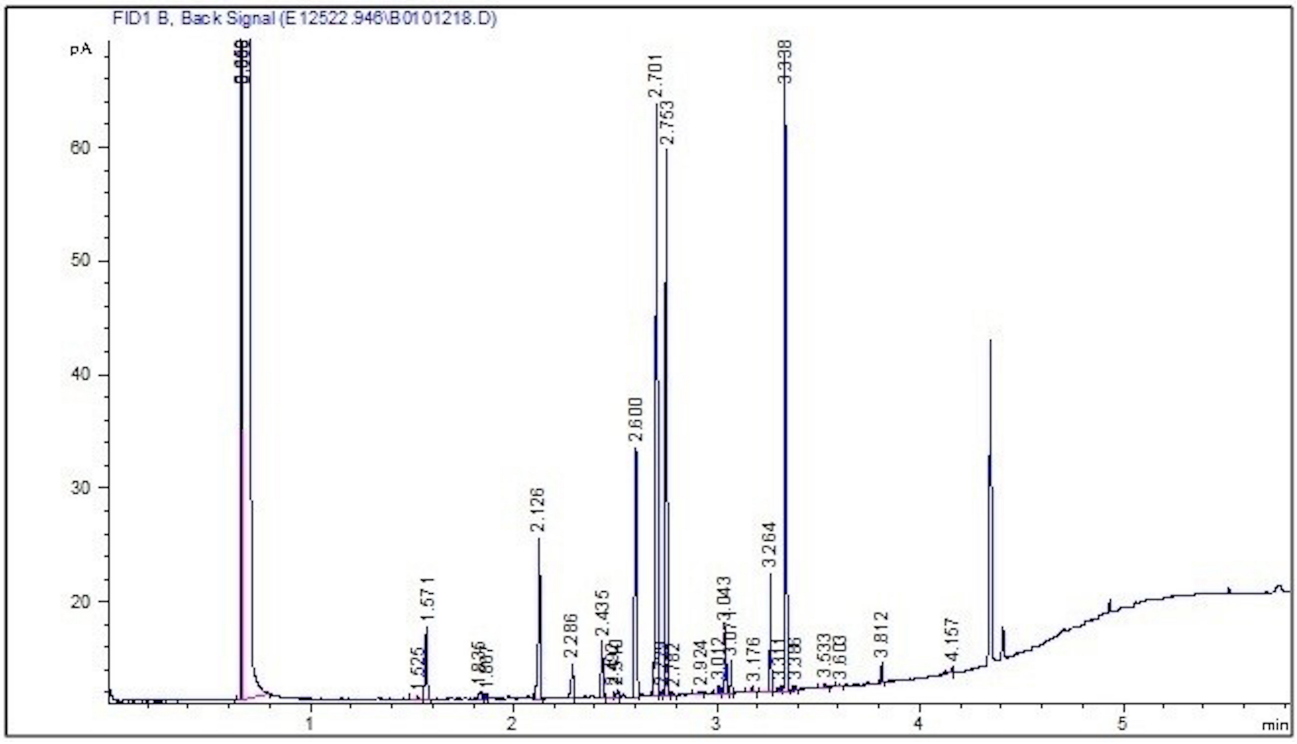

Fig. 2. Gas chromatography profile showing peaks of fatty acid methyl esters (FAME) for *Enterobacter* sp. CM94.

In addition, isolate CM94 had a unique fatty acid composition, with 14:0, 14:0 3OH/16:1 iso I, 16:1 ω7c/16:1 ω6c, 16:0, and 18:1 ω7c as the dominant forms (Fig. 2 and **Supplementary Table 3**). On the basis of the above fatty acid profile, the isolate provided its nearest match to the *Enterobacter* species, with a similarity index of >0.666 in the FAME-MIDI database.

3.5 Molecular Identification and Phylogenetic Analysis

A ~1.5 kb sequence of the 16S rRNA gene was obtained by the sequencing analysis. The BLASTn search results showed that the CM94 isolate had a 99.0% similarity with the existing 16S rRNA gene sequences of the *Enterobacter* species in the NCBI GenBank database. Based on the neighbor joining method, a phylogenetic tree of the CM94 isolate containing similar sequences from the NCBI database was created by 1000 bootstrap sampling (Fig. 3). The obtained 16S rRNA gene sequence (1440 bp) of CM94 was deposited into the NCBI database with the accession number: KC504003.

3.6 Effect of Rhizobacterial Isolate CM94 on Chickpea Growth-Promotion under Salt-Stress Conditions

Effects of the rhizobacterium CM94 inoculation in the mitigation of any adverse effects from salinity stress on chickpea growth was assessed under greenhouse conditions (Fig. 4). Results showed that all the salt stress treatments negatively affected the chickpea seedling growth in terms of a decrease in root length (RL), shoot length (SL), root and shoot fresh, and dry weight. However, isolate CM94 inoculation significantly ($p < 0.05$) increased the chickpea growth under all NaCl stress levels (Fig. 4). The maximum performance was observed at a NaCl stress of 75.0 mM. CM94 inoculation in 75.0 nm (T4) and 150.0 nm (T6)

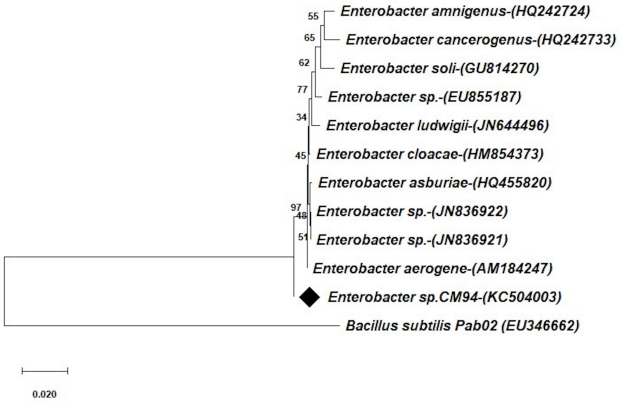

Fig. 3. Neighbor joining phylogenetic dendrogram based on 16S rRNA gene sequences showing relationships between CM94 and related taxa. *Bacillus subtilis* (EU346662) was used as an outgroup.

NaCl stress increased the root lengths by 41.6 and 38.2%, respectively, compared to the respective controls (T3 and T5) (Fig. 4). Likewise, salt stress reduced the shoot lengths by 29.4% (T3) and 74.8% (T5) at 75 mM and 150 mM NaCl concentrations, respectively, compared to the uninoculated control plants. However, with CM94 inoculation, increments of 37.4 and 84.3% were observed in shoot lengths under 75 mM and 150 mM salinities level respectively, compared to their uninoculated counterparts (Fig. 4). Comparisons to the uninoculated controls (T3 and T5) indicated a significant increase in the fresh roots (22.2 and 45.5%) and dry weights (29.7 and 80.7%) of the CM94-treated (T4 and T6) plants at the 75 and 150 mM NaCl concentrations, respectively. Similarly, inoculation with CM94 improved the shoot fresh weight (SFW) by 16.9 and 45.3% as well as enhanced the shoot dry weight (SDW) by 64.4 and 101.9% at salinities of 75 mM (T4) and 150 mM (T5), respectively (Fig. 4).

Determination of Chlorophyll, Proline, Sugar, and Protein Content

Chlorophyll content decreased by 21.1 and 49.6 % in the plants grown at 75 mM (T3) and 150 mM (T5) NaCl, respectively. Whereas increases of 23.7 and 31.6% were recorded in the chlorophyll content of the plants inoculated with the bacteria at 75 mM and 150 mM NaCl, respectively, compared to the respective uninoculated control plants (Fig. 5).

Also, significant increases ($p < 0.05$) in the proline contents of 67.9%, 14.5%, and 31.6% were recorded in the CM94-treated plants at 0 mM (T2), 75 mm (T4), and 150 mM (T6) salt-stressed conditions, respectively (Fig. 5).

Similarly, the sugar content was also found to be remarkably ($p < 0.05$) enhanced in the CM94-inoculated plants, compared to the control plants (Fig. 5). Inoculation of CM94 increased the sugar content by 44.6, 54.3, and 53.8%, compared to the uninoculated treatments *viz.* T1, T3, and T5. A substantial influence on protein content was observed with the treatment of *Enterobacter* sp. CM94 in chickpea seedlings grown under different levels of salinity stress. Here, it was detected that the bacterized seedlings showed a remarkable increase in the protein content of 60.4%, 72.4%, and 115.2%, compared to the uninoculated T1, T3, and T5 controls, respectively (Fig. 5).

3.7 MDA Contents

The effect of different salt stress with or without CM94 application on the lipid peroxidation levels of the chickpea plants was studied by measuring the MDA content. A significant ($p < 0.05$) increase in MDA content was obtained, which ranged from 107.1 to 220.3% as the salt stress increased from 75 to 150 mM. However, the application of the CM94 isolate remarkably decreased the MDA content in the plants. Results revealed that a reduction of 53% in MDA content at 150 mM NaCl stress levels followed by 45.1% at 75 mM, compared to the respective uninoculated plants (T3 and T5) (Fig. 6).

3.8 Effects of Enterobacter sp. CM94 on Antioxidant Enzyme Activities in the Plant

Modulation of antioxidant enzyme activities in response to CM94 inoculation was determined at 0, 75, and 150 mM NaCl stress (Fig. 6). The results demonstrated that as the salt stress increased from 75 mM (T-3) to 150 mM (T-5), all the antioxidant enzyme activities increased in the NaCl-treated plants compared to the uninoculated, non-stressed plants (T-1). Further, these antioxidant enzyme activities were additively increased by the bacterial inoculation (Fig. 6). The results showed that the activity of SOD increased by 25% and 38.9% in plants treated with 75 mM and 150 mM NaCl, respectively, compared to the uninoculated, non-stressed plants (T-1). However, compared to the uninoculated, stressed plants (T3 and T5), the CM94 isolate significantly ($p < 0.05$) increased the SOD activity by 45.6% and 54.3% in the same salt-treated plants (Fig. 6). Similarly, 75 mM and 150 mM NaCl stress enhanced the CAT activity by 8.9% and 26.1%, respectively, compared to the uninoculated control (T1). The application of the CM94 isolate significantly increased the CAT levels by 26.3% and 51% compared to the respective uninoculated salt-stressed plants (Fig. 6). An increase in salinity level from 75 mM to 150 mM also resulted in higher POD activities by 12.5% and 40.7%, compared to the uninoculated, non-stressed (T1) plants. Additionally, CM94 inoculation remarkably ($p < 0.05$) improved the POD activity by 112.8% and 124.1%, compared to their uninoculated, salt-stressed controls (T3 and T5) (Fig. 6). No significant variation was found between the CM94-inoculated and uninoculated plants under non-saline conditions, indicating that this antioxidant enzyme defense system is only activated under stress conditions (Fig. 6).

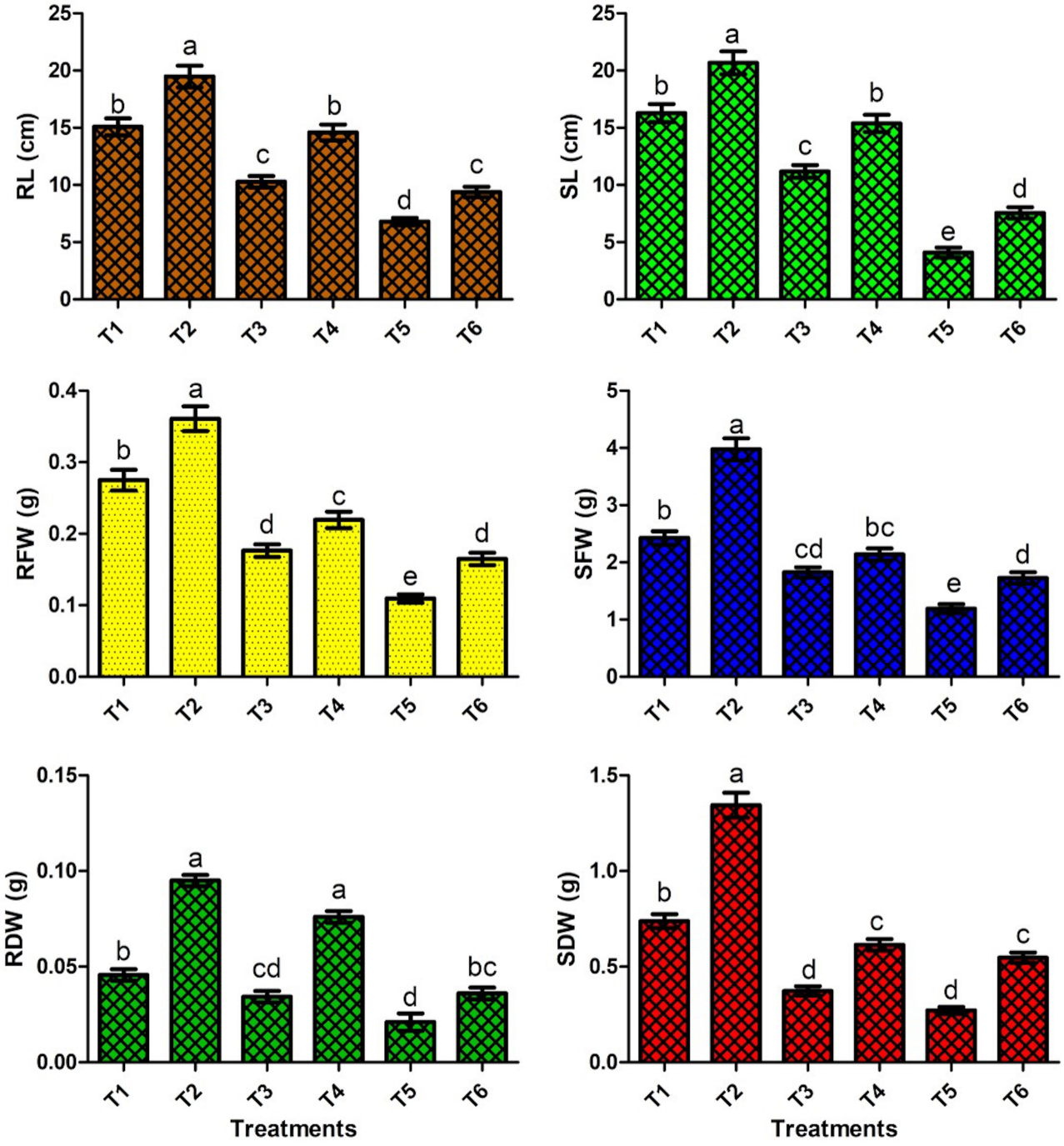

Fig. 4. Effect of *Enterobacter* sp. CM94 inoculation on chickpea growth under different NaCl conditions. T1: uninoculated control (no NaCl); T2: CM94 inoculation; T3: 75 mM NaCl; T4: 75 mM NaCl + CM94; T5: 150 mM NaCl; T6: 150 mM NaCl + CM94. RL, root length; SL, shoot length; RFW, root fresh weight; RDW, root dry weight; SFW, shoot fresh weight; SDW, shoot dry weight. The mean ± SE and n = 3 are presented. Different letters (a–e) represent statistical differences among different treatments. Where a and b are not very significant form each other in statistical sense. However, significant differences were found between a and c, a and d, a and e and between b and d, and b and e.

3.9 Root Colonization Assay

From the roots of inoculated plants (both stressed and non-stressed), bacterial colonies were obtained on the antibiotic-supplemented NA plates. Conversely, no rhizobacterial cells were grown from the root samples of the uninoculated plants. Further, to confirm the identity of the obtained bacterial isolates, ERIC-PCR-based DNA fingerprinting was performed. It was observed that the obtained

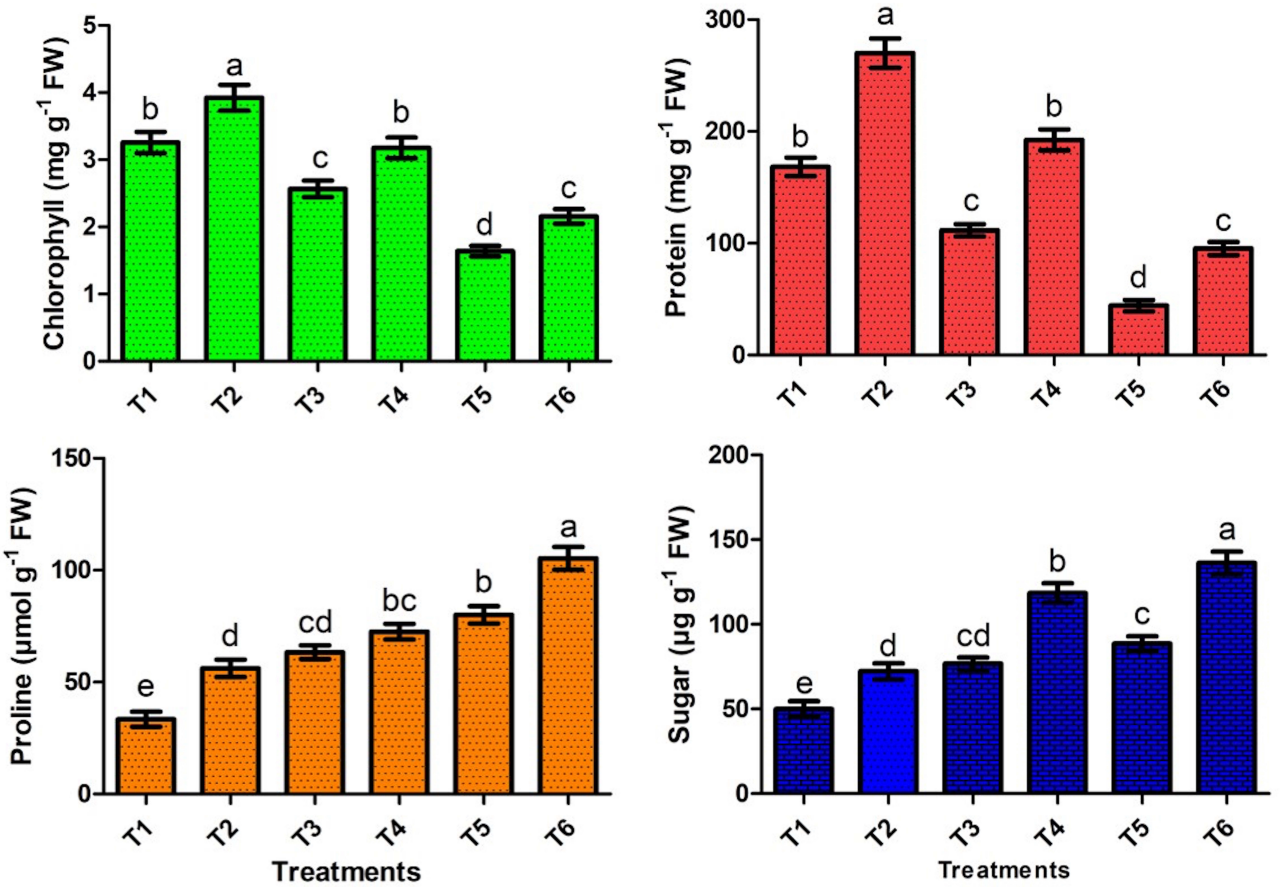

Fig. 5. Effect of *Enterobacter* sp. CM94 inoculation on chlorophyll, proline, sugar, and protein content in chickpea plants grown under different NaCl concentrations. T1: uninoculated control (no NaCl); T2: CM94 inoculation; T3: 75 mM NaCl; T4: 75 mM NaCl + CM94; T5: 150 mM NaCl; T6: 150 mM NaCl + CM94. The mean ± SE and n = 3 are presented. Different letters (a–e) represent statistical differences among different treatments. Where a and b are not very significant form each other in statistical sense. However, significant differences were found between a and c, a and d, a and e and between b and d, and b and e.

bacterial isolates from the inoculated root samples had the same ERIC-PCR banding patterns as the pure cultures of the wildtype CM94 isolate, thereby confirming the identity of the colonized bacteria (Fig. 7).

4. Discussion

A significant part of India's biological diversity is found in the fertile Indo-Gangetic Plain (IGP), along with the majority of its indigenous species. Additionally, the region has a complex geography with a large portion of infertile agricultural lands due to soil salinity. Improper management of the crops with indiscriminate and excessive application of synthetic chemical fertilizers further deteriorates crop production in such saline belts. In this context, PGPR has been considered as one of the possible alternatives for chemical fertilizers since they can mitigate the harmful effects of salinity in plants [47]. While screening novel isolates, it is crucial to consider their activity in a range of environmental conditions from where they are used. Therefore, it is of the utmost importance to study the indigenous rhizobacterial population in the same region where they will be employed as plant inoculants. Such climatic and temporal selection mechanisms will undoubtedly contribute to the evolution of a wide range of living beings, which are well adapted to shifting with climatic extremes. Thus, the search for salt-tolerant native rhizobacterial isolates in the present research work identified the salt-tolerant CM94 rhizobacterial isolate, which was isolated from the rhizosphere of chickpea plants grown in the salt-affected area of Uttar Pradesh. Isolated CM94 was able to tolerate and grow in conditions of up to 8% NaCl stress (Fig. 1). Hence, higher NaCl tolerances by CM94 in addition to PGP attributes can be used to enhance crop productivity in saline soils. The polyphasic characterization, based on the FAME (Fig. 2) and 16S rDNA sequence-based phylogenetic analyses, established the taxonomic position of the CM94 isolate as *Enterobacter* sp. (NCBI Accession Number: KC504003) (Fig. 3). Earlier studies showed that the rhizobacterial genus, such as *Bacillus* tended to be predominant in IGP saline soils [15]. However, less attention was provided to other genera, meaning they need to be further explored. *Enterobacter* species are an exceptionally diverse

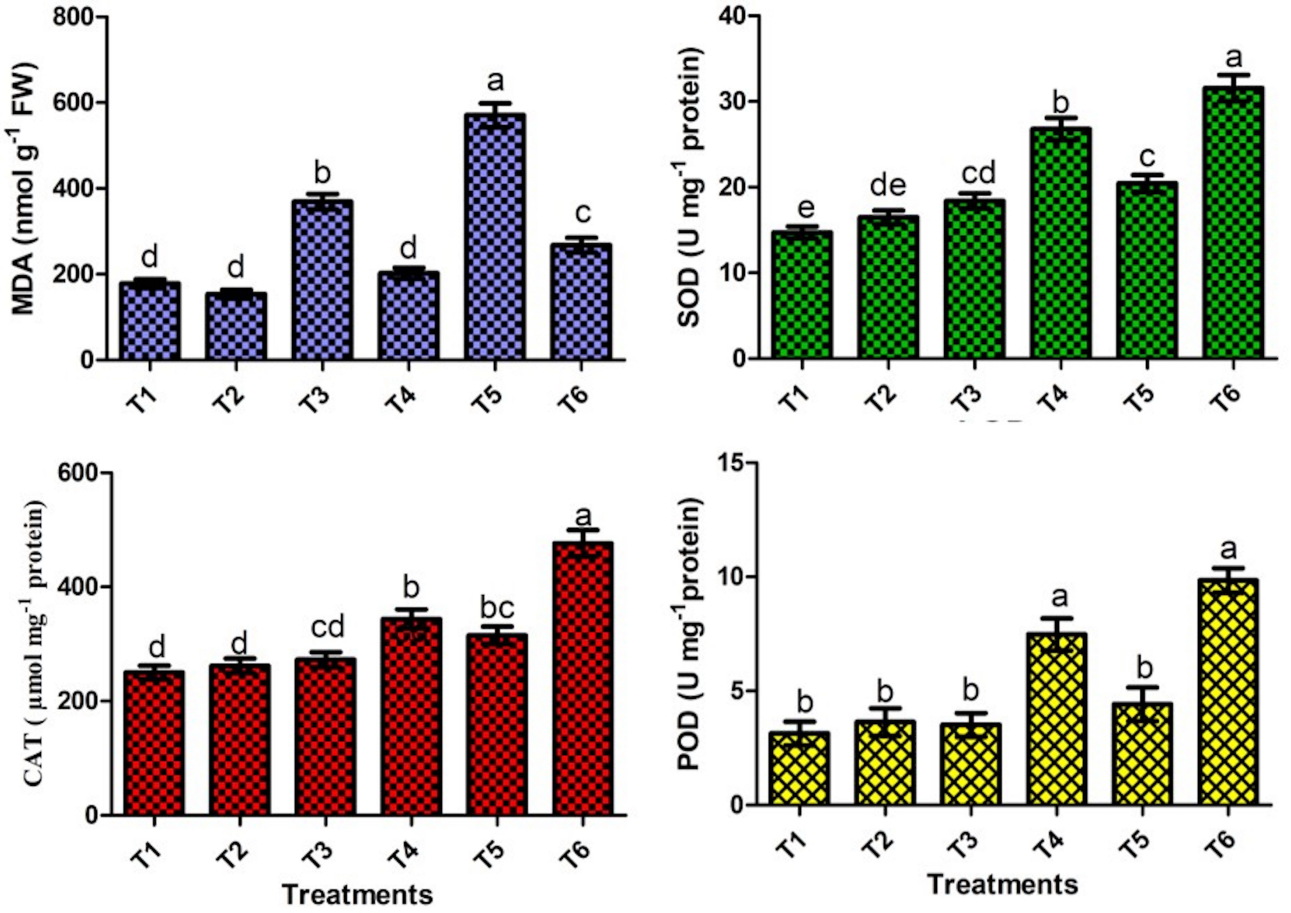

Fig. 6. Effect of *Enterobacter* sp. CM94 inoculation on MDA and antioxidant enzyme activities in chickpea plants grown under different NaCl concentrations. T1: uninoculated control (no NaCl); T2: CM94 inoculation; T3: 75 mM NaCl; T4: 75 mM NaCl + CM94; T5: 150 mM NaCl; T6: 150 mM NaCl + CM94. The mean ± SE and n = 3 are represented. Different letters (a–e) represent statistical differences among different treatments. Where a and b are not very significant form each other in statistical sense. However, significant differences were found between a and c, a and d, a and e and between b and d, and b and e.

group of organisms that exhibit high levels of metabolic and genetic diversity. These bacterial groups possess multifarious plant growth-promoting traits, including the production of IAA, ACC deaminase, siderophore, ammonia, and HCN, as well as potentially improving the availability of important nutrients, such as phosphate, thereby making these bacteria very useful in the agriculture field [18,21].

IAA is an important signaling molecule in microbe–plant interactions. IAA regulates cell division and lateral root elongation and has a major role in stress responses [48]. Soil salinity adversely affects the production of IAA in the roots and leaves of plants [49]. As a result, the reduced levels of IAA in the plant roots remarkably hampers the overall growth and development of plants. However, under salt stress conditions, some PGPR can synthesize IAA, which in turn, can enhance the root architecture and plant biomass [50]. Phytohormones, synthesized by microorganisms, induce the development of roots with higher surface area and biomass, which enables plants to extract more nutrients from saline soil. Masmoudi *et al*. [51] reported that the IAA producing *Bacillus velezensis* FMH2 significantly enhanced root length and lateral root production, and enhanced tomato plant growth under salt stress conditions. In congruence to this, the CM94 isolate also improved root length and biomass, which might be mainly attributed to IAA production.

ACC deaminase cleaves ACC (the immediate precursor of ethylene) into ammonia and α-ketobutyrate, which in turn can serve as a source of N and C for bacteria. Thus, ACC deaminase-producing bacteria lower ethylene levels in the plant during stress conditions [22]. In the current study, consistent ACC deaminase activity in the CM94 isolate under various ranges of salt stress showed its potential to promote plant growth under stress conditions. Inoculation with such ACC deaminase-producing PGPR will help to reduce the detrimental effects and sustain plant growth under stress conditions. Earlier studies also reported that salt-tolerant ACC deaminase-producing rhizobacteria could manage ethylene levels, and thus, improve stress tolerance and growth of plants [52]. In this study *Enterobac-*

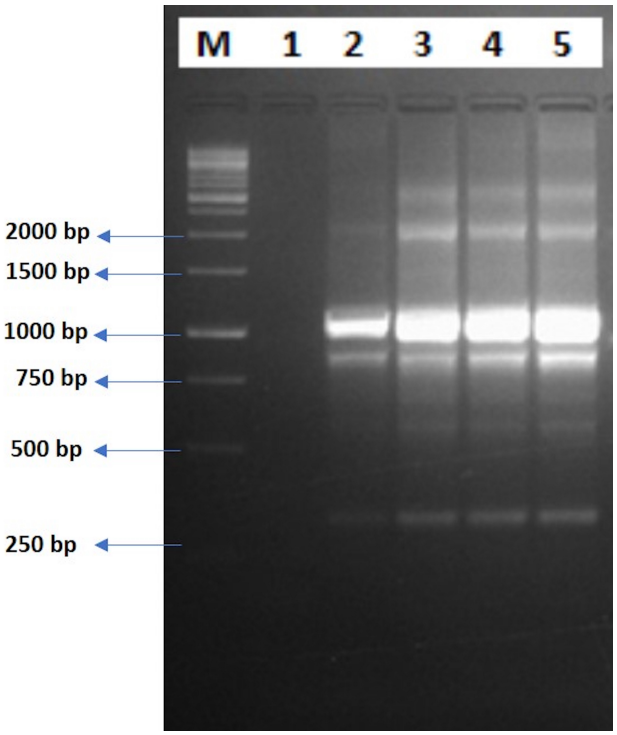

Fig. 7. Confirmation of ERIC-PCR-based root colonization. Lane 1: 'M': 1000 bp marker; lane 2: negative control; lane 3: wild CM94 isolate; lane 4: isolate obtained from 0 mM NaCl-treated CM94-inoculated plant (T2); lane 5: isolate obtained from 75 mM NaCl-treated CM94-inoculated plant (T4); lane 6: isolate obtained from 150 mM NaCl-treated CM94-inoculated plant (T6).

ter sp. CM94 was able to produce EPS at all tested NaCl concentrations, with the maximum at a NaCl concentration of 4%. It is well documented that under stress conditions, the production of EPS is one of the most vital responses of PGPR to combat stress. It has been documented that bacterial EPS could bind Na^+ and, therefore, increase plant growth by restricting Na^+ uptake by the plant's roots, which helps manage the K^+/Na^+ equilibrium [8].

Nutritional imbalance is known to be a salinity-induced adverse effect on plant growth and production. Salt stress has adverse effects on P- uptake and transport in the plant. P fertilizers are generally suggested to be used for managing P deficiency in salt-affected soils. The use of salt-tolerant P-solubilizing-rhizobacteria can greatly improve P availability in saline soils. In the present study, isolated CM94 was found to be a highly efficient solubilizer of insoluble phosphates. The results are in accordance with earlier studies, where salt-tolerant PGPR strains representing various genera, such as *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Burkholderia*, *Pantoea*, and *Serratia*, were able to solubilize phosphate and improve the growth of the plants [15,53]. Furthermore, salt stress is a cause of iron deficiency and together they could impose a negative effect on the plant's vegetative growth, stomatal conductance, photosynthesis, and transpiration rates [54]. Finding prospective siderophore-producing PGPR that are also salt-tolerant may be a beneficial non-transgenic method for reclaiming the salinity-affected soils for agriculture. In the present study, the CM94 isolate could produce a significant amount of siderophore, which was retained even at higher salinity levels. Thus, the siderophore-producing ability of microorganisms under stress conditions may be an auspicious alternative to chemical fertilizers and could aid in managing salt stress and iron limitations in salt-affected soils. Recently, a study by Sultana *et al*. [55] reported that salt-tolerant siderophore-producing PGPR supported rice growth.

The application of PGPR is one of the most prospective approaches to alleviate salt stress in plants. When isolated CM94 was evaluated for growth promotion in chickpea plants in the greenhouse, it displayed a significant increase in root length, shoot length, and biomass. Particularly, increased root, shoot length, and biomass are very important for improved nutrient uptake and are also essential for coping with different environmental stresses, including salinity, which is a serious problem in the IGP region of U.P. India. Li *et al*. [56] reported that salt-tolerant *E. cloacae* HSNJ4 significantly promoted vegetative growth, and improved photosynthetic pigment content, and salinity tolerance by balancing the relative IAA and ethylene content in plants. The findings in the present study are also in line with the earlier reports where the use of salt-tolerant PGPR, such as *Sphingomonas*, *Bacillus*, and *Enterobacter* enhanced the growth, development, and yield of plants under saline conditions [8,9,14,21]. It might be due to improved nutrient uptake and water absorption, which helped plants cope with the salinity stress. One of the possible mechanisms for mitigating the salt-stress symptoms is the production of Na^+-binding EPS, enhanced ion homeostasis, improvement in uptake of essential nutrients (including P), synthesizing phytohormones, and decreasing ethylene concentrations through ACC deaminase [57].

In addition, an improvement in the physio–chemical parameters of plants was also observed in CM94-inoculated plants. The leaf chlorophyll content represents a primary indicator of photosynthesis, which correlates well with salt tolerance [58]. Under salt stress conditions, CM94-inoculation remarkably increased the chlorophyll content, compared to the respective uninoculated plants, which indicated an improvement in the photosynthetic activity. A similar increase in the plant's photosynthetic activity and chlorophyll content under salt stress was also reported in earlier studies [59]. An improvement in photosynthetic activity is also one of the important contributors to enhancing shoot biomass.

Furthermore, in response to salinity stress, the accumulation of compatible solutes, such as proline, is a common stress-avoidance mechanism that guards the plant's cell membrane, scavenges the free hydroxyl radicals, and stabilizes the structure of the proteins [60]. In addition, during NaCl stress, soluble sugar together with proline as-

tonishingly maintained the osmotic equilibrium and formed around 50% of the total osmotic potential in plant cells [61]. Along with proline, soluble sugars, by making up 50% of their total osmotic potential, help plants maintain their osmotic homeostasis under NaCl stress. In the present study, inoculation of CM94 in chickpea plants enhanced the production of proline and sugar, which could significantly contribute to mitigating the salinity stress. Our results are in accordance with earlier studies, which also reported that the application of the *Bacillus* species improved the salt-stress tolerance and growth of wheat plants by modulating transpiration, photosynthesis, and proline accumulation [62,63]. Furthermore, in PGPR-inoculated plants, the protein content was also increased under stressed and non-stressed conditions. The results are in congruence with the study by Sultana *et al*. [55], who reported that inoculation with the *Pseudomonas* strain provided differential protein concentrations in rice plants. The higher protein content is also related to improved photosynthesis, which in turn represents a function of higher chlorophyll content.

The malondialdehyde (MDA) concentration is also an indicator of oxidative injury under salinity stress and is directly related to the plant's lipid peroxidation [64]. In the present work, it was observed that CM94 inoculation reduced the MDA level in stressed plants, compared to the uninoculated control plants. Reduced MDA levels by PGPR inoculation is an indication of a decrease in cell membrane damage, which enhances salt stress tolerance in plants. The results are consistent with the study by Singh and Jha [65], who reported that PGPR inoculation remarkably reduced the MDA content and alleviated lipid peroxidation in plants under stressed conditions.

Salt stress induces an unavoidable consequence of oxidative stress and generates a higher amount of ROS, which poses a severe threat to the plant's cells by triggering lipid peroxidation, protein oxidation, nucleic acids, and enzyme damage [66]. To detoxify excess ROS from cells, plants exhibit excellent antioxidant enzymes, such as SOD, CAT, POD, APX, etc. These antioxidant enzymes can scavenge the free radicals produced during oxidative stress [67]. Activation of SOD leads to the conversion of O^{-2} into H_2O_2 in the chloroplast, mitochondrion, cytoplasm, apoplast, and peroxisome. Further, this accumulation of H_2O_2 activates the CAT and POD enzymes, which diminish its concentration by splitting it into O_2 and H_2O [68]. Activities of the antioxidant enzymes, such as SOD, CAT, and POD, were increased in chickpea plants inoculated with PGPR compared to uninoculated ones, under both 75 mm and 150 mM NaCl stress levels (Fig. 6). Notably, the results confirmed that CM94-treated plants adapted to salinity stress conditions by removing ROS through an increase in antioxidant enzymatic activities. The results of the present study are also in line with the findings of Habib *et al*. [69], Vaishnav *et al*. [9], and Shultana *et al*. [70], who reported that the PGPR inoculation increased the ROS-scavenging antioxidant enzymatic activities in okra and tomato plants under salt stress conditions.

Generally, the benefits of PGPR inoculation depend on its effective root colonization. Therefore, in the present study the colonization efficacy of the potential PGPR isolate CM94 was assayed following antibiotic-resistant and ERIC fingerprinting approaches. The initial and most important stage of a plant-microbe relationship is root colonization, during which bacteria travel toward the rhizosphere in response to root exudates. Antibiotic-resistance markers can be particularly helpful for colonization studies of PGPR isolates. In the present study, by using the combination of antibiotics amended in NA, the inoculated PGPR CM94 isolates were retrieved. Contrastingly, no bacterial colony was obtained from the uninoculated plants. These results showed that CM94 efficiently colonized chickpea roots. Further, the identity of the colonized bacterial isolates was confirmed by comparing the DNA fingerprints of wildtype PGPR CM94 isolate and the root colonized bacteria by ERIC-PCR. The results of the present study are in accordance with the study by Singh *et al*. [65], where they proved bacterial colonization through ERIC-PCR DNA fingerprinting analysis.

5. Conclusions

The results of the present study revealed that *Enterobacter* sp. CM94, with a salinity tolerance, possesses multiple plant growth attributes, which are even retained at higher salt concentrations. Inoculation with the CM94 isolate alleviated the salt stress and induced systemic tolerance in chickpea plants by significantly improving their growth and biochemical parameters as well as the expression of ROS-scavenging antioxidant enzymes. The results demonstrated that the utilization of such PGPR would enable a decrease in the use of chemical fertilizers, which will in turn help to achieve sustainable production of chickpea plants in the saline soils of eastern Uttar Pradesh, India. However, future studies are required to determine the nature of the isolate under different salt-affected areas and to harness their inherent positive capability as bioinoculants in farmer's fields.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

AS, RK, and NK, conceptualized the idea and designed the experiments. AS, executed all the laboratory experiments, analyzed the data, and wrote the manuscript. AV, helped in software and data analysis. HC and AKS, helped in methodology and validation of results. YKB, helped in formal analysis, visualization, and investigation.

NK and RK, helped in formal analysis, funding acquisition, writing, review, and editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The chickpea plant seeds used in this study were procured from ICAR-Indian Institute of Seed Science (IISS), Mau Nath, Bhanjan (U.P.), India.

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Conflict of Interest

The authors declare that there is no conflict of interest. Given the role as Guest Editor and Editorial Board Member, Naeem Khan had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Jorge M.L. Marques da Silva.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2810241.

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