ACC deaminase producing bacterium *Enterobacter cloacae* ZNP-2 mitigate salinity stress and enhance salinity stress tolerance in wheat plant

Ayushi Sinha[1], and Rajnish P. Singh[1]*

[1]Department of Biotechnology, Jaypee Institute of Information Technology, Noida, Uttar Pradesh, India

Abstract Increasing soil salinity adversely affects plant growth and productivity. However, some salt-resistant rhizosphere bacteria have the possible ability to increase the growth of plants when put under saline stress conditions. Among ACCD (ACC deaminase)-producing bacteria which have been extracted from the rhizosphere of plants growing in salt-rich desert soil, ZNP-2 was selected based on its ability to produce phytohormone and ammonia, and solubilize phosphate, and further identified as *Enterobacter cloacae*. Furthermore, we evaluated the protective effects of the inoculation of *Enterobacter cloacae* ZNP-2 on morphological and physiological growth parameters, ionic balances, accumulation of osmolytes, and antioxidative defense system under both normal and salt stress conditions. Through polymerase chain reaction, the presence of AcdS gene, which is responsible for the structural gene for ACCD was confirmed for the strain ZNP-2. The increase in saline stress leads to accumulation of toxic Na^+ and decrease in levels of K^+, thereby favouring the K^+/Na^+ ratio. Moreover, ZNP-2-inoculated plants showed improvements in biomass (13% to 31%) and chlorophyll contents (25% to 51%) as compared to the un-inoculated plants. ZNP-2 inoculation also improved the various osmolytes in wheat plants to maintain the osmotic balance. The observation implies that ZNP-2 isolate augments salt tolerance in wheat plants by modulating the intracellular level of various osmolytes. Therefore, the utilization of beneficial microbial isolate as a mechanism for inducing salt tolerance in wheat plants could be used as an effective tool to combat salt stress in plants.

Keywords ACC deaminase • Osmolytes • AcdS • IAA • PGPR

1. Introduction

Plants in their natural environment are confronted with various kinds of biotic (pest, weed, disease, etc.) and abiotic (salinity, drought, high temperature, pollutants, etc.) stressors, the plant's growth and maturation is greatly hindered by these stressors. Out of these stressors, one of the major environmental stressors is increasing soil salinity which deleteriously affect harvest yield around the world, consequently affecting the food security [1]. Salinity affects a broad range of physiological functions such as ionic homeostasis disruption, photosynthesis, nitrogen fixation and other metabolic processes in the plants responsible for osmotic adjustment [1,2]. A negative impact has been observed in the overall plant growth whose major cause has been observed to be abnormal production of 'stress ethylene' [3]. In order to minimise the ethylene-induced growth inhibition, an effective approach of decreasing elevated 'stress ethylene' levels within the plant can be used [4]. Plant productivity is significantly limited because of enhanced ethylene levels under various biotic and abiotic stresses such as salt, pathogens attack, flooding, drought, metals, and organic contamination [5]. Additionally, plants growing under stress conditions like salinity face insufficient levels of micro and macronutrients, lack of sufficient water supply, physicochemical effects of soil, which further leads to a reduction in the yields of the crops grown worldwide. The use of pesticides may partially overcome these constraints, however, their continuous use in the soil reduces the essential nutrients. Under these unfavourable conditions, amelioration of these constraints by biological method is highly desirable.

Previous studies have shown that bacteria with ACCD activity maintain plant growth under diverse range of stressors by reducing the extent of ethylene levels which are induced due to stress [6]. Cleavage of the α-ketobutyrate and ammonia from immediate precursor of ethylene 'ACC' (1-aminocyclopropane-1-carboxylic acid is a major mechanism of ACCD bacteria [7]. Aside from ACCD activity, the plants also benefit from various microbial activities, such as, siderophore production, ammonia production, phytohormone production, nitrogen fixation, inorganic material (phosphate) solubilization, antibiotic production, and lytic enzyme secretion, etc. It is well-established that these bacteria are useful for promoting plant growth in a variety of stressful environments, including those caused by metals, salt, and organic pollutants [8].

Attempting to reduce ethylene levels with the use of bacteria inoculums can help plants to withstand various environmental stressors. A phenomenon known as 'induced systemic tolerance' is observed in plants when plant growth promoting bacteria induce physiochemical changes which lead to enhanced tolerance [9]. For sustainable growth and development of plants under challenging condition, ACCD-producing bacteria, phytohormones, and various metabolites produced by host-associated PGPR (plant growth promoting rhizobacteria) are essential for promoting growth and maintaining proper nutrition balance [10]. Previous studies have demonstrated the alleviation of abiotic stress responses following inoculation of PGPR in different crop plants [11].

Cellular Na^+ toxicity generated through increased salinity disrupts the photosynthetic processes, resulting in a reduction of carbon fixation and biomass production in plants [12]. K^+ and Ca^{2+} uptake by plants are decreased due to the presence of salinity-like stressors which increases Na^+ uptake and therefore generates the metabolic abnormalities [13]. However, under

these circumstances, host-associated PGPR (plant growth promoting rhizobacteria) support the plant growth by decreasing Na^+ levels and increasing K^+ levels, therefore favouring the K^+/Na^+ ratio. Several previous studies have reported various PGPR strains reducing the salinity-induced damage in a wide range of crops by favouring K^+/Na^+ ratio in saline stress conditions [14]. A study by Zhang et al. showed that *Bacillus subtilis* (GB03) enhanced the salt tolerance of Arabidopsis through the regulation of Na^+ by modulating the expression of AtHKT1 [15]. Another study by Singh et al., reported that Klebsiella sp. SBP-8 protected the wheat plants under saline stress by regulating Na^+ and K^+ levels, especially favouring K^+/Na^+ ratio [16].

The accumulated reactive oxygen species (ROS) caused because of soil salinity causes oxidative stress on plant cells. Overproduction of ROS agents like O_2^-, H_2O_2, and OH^- (superoxide, hydrogen peroxide, hydroxyl radicals) which imposes several cellular damages through proteins, lipids, and nucleic acids oxidation [17]. These induced-ROS target the membrane phospholipids which can mediate membrane damage and increase lipid peroxidation [18]. ROS overproduction in plants can possibly avoided by triggering the antioxidative scavenging mechanisms to minimize structure damage. Previous study documented a beneficiary relationship among the salinity resistance and antioxidative defence system [19]. In order to counteract the impact of salt stressors, PGPB also induce the antioxidative defence machinery to a significant level [20]. Following salt stress, many plants accumulate compatible solutes or osmolyte solutes like sugars, polyamines, betaines, proline, and other amino acids that serve as a cytoplasmic osmoregulation for inhibiting the effects of salts on membrane proteins and their integrity [21]. Several macromolecular structures are protected in case of physiological drought induced by salinity because of the accumulation of osmolytes which maintains the turgor pressure. Following the various environmental stressors such as salinity, the level of these solutes is highly variable and significantly increases [22]. It is assumed that beneficial bacteria enhance osmotic-stress tolerance by increasing the levels of endogenous Osmo-protectants [23]. Moreover, deeper understanding of compatible solute mediated specific mechanisms of resistance to various abiotic stressors is still limited.

Wheat is an important crop plant, whose germination and growth can generally be stopped by saline stress. Nawaz et al. (2020) reported the elevated salt levels significantly reduced seed germination and also caused poor root growth in wheat. Therefore, sustainable management options are required to minimize the effects of abiotic stressors. Currently salt-tolerant PGPB are considered potential bioinoculants to enhance crop productivity in saline agriculture [24]. The aim of the current research was to isolate bacteria that produce ACCD from plants that are developing in saline soil of deserts of Rajasthan, India. The selected isolate was further tested for various PGP traits like production of growth hormone, antagonistic activity, HCN production, phosphate solubilization, etc. We assessed bacterial inoculation impact on morphological and physiological development parameters, ionic balance, osmolyte accumulation, and the antioxidative defence system in normal and stress conditions using pot experiments. Current study was an effort to understand the stress-mitigating mechanism of an ACCD-producing bacterium, which can be used as development of biofertilizers for plants growing under salinity stress.

2. Material and Methods

2.1 Isolation of rhizospheric bacteria and measurement of ACCD activity

The rhizospheric soil sample of *Ziziphus nullifera* was collected from Rajasthan, India's deserts. The soil & five to six crop plants were removed and transported to lab, stored in zip-lock bags for additional testing. Serial dilution plating method in Luria-Bertani (LB) supplemented with 1.8% agar was used to isolate the rhizobacteria from the soil. An LB-agar plate was plated with 100 µl of the proper dilution, and was incubated for 24 hours at 37°C. The morphological different colonies were further plated on DF (Dworkin and Foster) salt media supplemented with 3 mM ACC besides of $(NH_4)_2SO_4$ as sole nitrogen source [25]. As per standard protocol, the DF-ACC plates were inoculated and incubated at 30°C for 3 days, after which the grown colonies were subjected to ACCD production. The ACCD production was quantified by evaluating α-ketobutyrate (KB) quantities which are produced after ACC hydrolysis. KB quantities produced because of microbial ACC was measured at a wavelength of 540 nm in relation to α-ketobutyrate standard curve (0.1 to 1.0 µl). Following ACCD-production, one colony marked as ZNP-2 was tested for other PGP traits. The selected isolate was preserved in the glycerol (15%) solution and stored at -80 °C for further assay.

2.2 Phylogenetic analysis

The Qiagen Bacterial DNA Isolation Kit was used to extract ZNP-2's genomic DNA. The universal primers 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-TACGGCTACCTTGTTACGAC-3' were used to amplify the 1.5 kb region of 16S rRNA. At Eurofins Genomics India Pvt. Ltd. (Bangaluru, India), the PCR product was further sequenced after being examined on 1% agarose gel. The phylogenetic tree of the sequence was constructed by NJ-method using the tool MEGA-X with a boot strap of 1000 replicates [26].

2.3 Amplification of AcdS gene

Using universal primers, the AcdS gene, which codes for ACCD, was amplified by PCR. Using 50 ng of genomic DNA, forward and reverse primers (20 picomole each), dNTP mixtures (200 µM each), 1X Taq polymerase buffer, and 2.5 U of Taq DNA polymerase (Genei, India), a PCR reaction was conducted in a 25 µl reaction volume. In Bangalore, India, at Eurofins Genomics Pvt. Ltd., the amplified AcdS gene was sequenced. The BLAST-N search program (http://www.ncbi.nlm.nih.gov)

was used to analyse the obtained gene sequence for nucleotide sequence homology. The CLUSTAL-X program was used for aligning the sequence and NJ-tree (Neighbour-Joining) was constructed with bootstrap value of 1000.

2.4 Test for other PGP traits

2.4.1 Auxin Production Assay

The amount of IAA produced by the chosen ACCD-producing isolate ZNP-2 was measured at 530 nm by adding 100 µg/ml of L-tryptophan to the media. The mixture was incubated for three days at 200 rpm and 30°C. The IAA production was assessed by the colorimetric method using the Salkowski reagent method and the appearance of development of red color was measured at 530 nm by a UV-Vis spectrophotometer [27]. The quantity of produced IAA was determined with a standard curve of pure IAA (Sigma-Aldrich, USA) ranging between 0 to 100 µg/mL [28].

2.4.2 Phosphate solubilization

Isolate ZNP-2 was tested for phosphate solubilization ability using NBRIP medium [Composition: g/L 10, glucose; 5, $Ca_3(PO_4)_2$; 1, $MgSO_4.7H_2O$; 0.2, KCl; 1, NaCl; 5, NH_4Cl; and 2% agar, pH 7.0] [29]. The amount of free phosphate released was measured by comparing it to a standard curve of different K_2HPO_4 concentrations.

2.4.3 Siderophore production and nitrogen fixation test

The siderophore production was determined by the Chrome-Azurole-S (CAS) assay. After being spot-inoculated on a CAS-agar plate, ZNP-2 was incubated at 30 °C for 4-5 days. The change in color (orange/yellow) around the bacterial growth indicates siderophore production [30]. The test for nitrogen fixation was evaluated by growing the isolate on N-free JNFb medium [31].

2.4.4 NH_3 and HCN production

The estimation of ammonia production was verified using Nessler's reagent to the bacterial culture grown in peptone water broth (peptone 10g/L, NaCl 5g/L). The appearance of a brown to yellow colour was an indication of ammonia production [32]. Isolate was tested for HCN production by the filter paper method involving an alkaline picric acid solution (2% Na_2CO_3 in 0.5% picric acid) and 0.4% (w/v) glycine [31].

2.4.5 In-vitro assay for stress tolerance

The tested isolate's growth was observed for 72 hours at 30°C in LB-broth medium supplemented with 2 to 10% NaCl in order to assess its capacity for salt tolerance. Likewise, the isolate's growth was examined at varying pH values (2-12). A UV-Vis spectrophotometer was used to measure the culture's cell suspension density at 600 nm for each treatment. Uninoculated medium was used as a blank for measurement, which was carried out in triplicate.

2.5 Motility Test

Isolated bacterial strain was screened for its swimming, swarming, and twitching behavior. The bacterial isolate was point-inoculated onto swim agar plates supplemented with 0.3% agar, tryptone (1%), and NaCl (0.5%) for swimming, and then incubated for 16 hours at 25°C. The isolate was spot inoculated on nutrient broth (0.8%) that contained 0.5% bacto-agar for the swarming assay. It was then supplemented with 0.5% dextrose and incubated for 24 hours at 30°C. Stabbing inoculated to the bottom of the petri dish on an LB-agar plate (1% agar) allowed for the monitoring of twitching behaviour.

2.6 Effect of ACCD-producing strain on plant growth

The plant growth promotion under normal and saline stress (150 mM, 175 mM, 200 mM NaCl) was performed in an environmentally controlled growth chamber. By determining the morphological, biochemical, and ionic balances of wheat plants, isolate ZNP-2 was evaluated for its capacity to promote growth and reduce the adverse effects of salt stress. To induce ACCD activity, strain ZNP-2 was prepared in DF-ACC minimal medium and incubated for 3 days at 200 rpm at 30°C. The cells were harvested by centrifugation at 12,000 g for 15 mins and cell pellets were suspended in 0.03 M $MgSO_4$ to make the final OD of 0.15 at 590 nm [28].

The seeds of the wheat (*Triticum aestivum* L.) plant were properly sterilized with washing in 70% ethanol (v/v) and further sterilized with 1% sodium hypochlorite (NaClO) solution for 2 mins. To get rid of any remaining bleach solution, the seeds were lastly rinsed five or six times with sterile deionised water. Under aseptic conditions with laminar air flow, seeds were bacterised by immersing them in the corresponding bacterial suspension for one hour. Unprimed wheat seeds submerged in a 0.03 M $MgSO_4$ solution made up the control group. Each treatment in the in-vitro pot study was replicated five times using a randomised block design (RBD). The physiochemical constituents of soil were determined by Atomic Absorption Spectroscopy (AAS 2380, Perkin Elmer, USA) and were pH (6.98), total N (0.19%), available P (10.8 mg/kg), extractable K (130 mg/kg), and organic carbon (0.71%). To prevent the presence of any bacteria or spores, the soil was autoclaved for three days in a row at 15 psi and 121°C. Plastic pots were filled with the sterilized soil. Five seeds were sown in each pot, and the germinated seedlings were placed using sterile forceps. Up to 15 days after seed germination, pots were kept in a growth chamber with 16:8 photoperiods, 60% relative humidity, and light intensity of 400 $Em^{-2}s^{-1}$ (400-700 nm) at 24 ± 2°C. A total of four

treatments were compared to evaluate the response of ZNP-2 on the wheat plants growing under imposed salinity stress. The treatments were: T0; control plants with and without bacterial inoculum, T1; plant treated with 150 mM salt stress with and without bacterial inoculums, T2: plant treated with 175 mM salt stress with and without bacterial inoculums, T3: plants subjected to bacterial inoculums and 200 mM salt stress. The salt stress was imposed using Hoagland medium supplemented with the appropriate concentration of NaCl (150 mM to 200 mM). Following the experiment, plants were carefully removed from the pots, and ten randomly chosen seedlings from each replication were measured for growth parameters, including shoot and root lengths, fresh and dry weights, chlorophyll content. For chlorophyll estimation, 0.5 g leaf samples from each replicate were homogenized in 80% acetone and pigments were extracted and quantified at 480, 510, and 663 nm in a UV-Vis spectrophotometer [33].

2.7 Ionic Analysis

For ionic analysis, plants in each treated group were carefully washed with sterilized deionized water four to five times to remove any adhered soil aggregates and kept for oven dry at 70 °C for 48 h. Afterwards, 1g of plant tissue was homogenized in liquid N_2 and further digested in a mixture of H_2O_2 (30%), HNO_3 (65%) and deionized water in a 1:1:1 ratio at 120 °C for 2 h. The final volume was made up to 20 ml and ions mainly, Na^+, K^+ and Ca^{2+} in the shoot tissue were determined at the NHRDF (National Horticultural Research and Development Foundation, Nashik, India) in triplicate sets [16].

2.8 Biochemical analysis

2.8.1 Proline content analysis

The proline content in fresh wheat leaves was measured by spectrophotometric absorption at 520 nm following the standard procedure of Bates et al. and expressed as μmol g^{-1} fresh weight [33]. Measurement of lipid peroxidation was determined by the thiobarbituric acid (TBA) reagent method following its molar extinction coefficient (155 $nm^{-1}cm^{-1}$) [34]. The total soluble sugar was measured in both normal and saline-treated plants following the phenol-sulfuric reagent method [35].

2.8.2 Colonization

The isolate's colonization was tested using serial dilution method. To put it simply, 1g of root segments were ground into 1X sterilized PBS buffer, and the resulting bacterial suspension was plated on LB-agar to count the number of bacteria. The results were expressed in colony-forming units (CFU).

2.8.3 Statistical Analysis

A two-way ANOVA was performed on all PGP trait data in order to examine the impact of the experiment's treatments. Additionally, the significant difference was examined using LSD (least significant difference, p=0.05).

3. Results

3.1 Isolation and identification

Five bacterial isolates were obtained from the rhizospheric soil of Ziziphus nullifera after they showed steady growth on the DF-ACC agar plate. Here, we describe the findings for isolate ZNP-2 based on the increased production of ACCD. Following measurement, the ACCD was 160.30±17 nmol of α-KB mg^{-1} protein (Pr.) h^{-1}. The 16S rRNA gene was amplified in order to determine ZNP-2's taxonomic affiliation at the molecular level. A sequence comparison revealed that ZNP-2 was 99% similar to Enterobacter cloacae and other Enterobacter sp. (Fig 1). After being submitted to the NCBI Genbank, the sequence was given the accession number KJ950703.

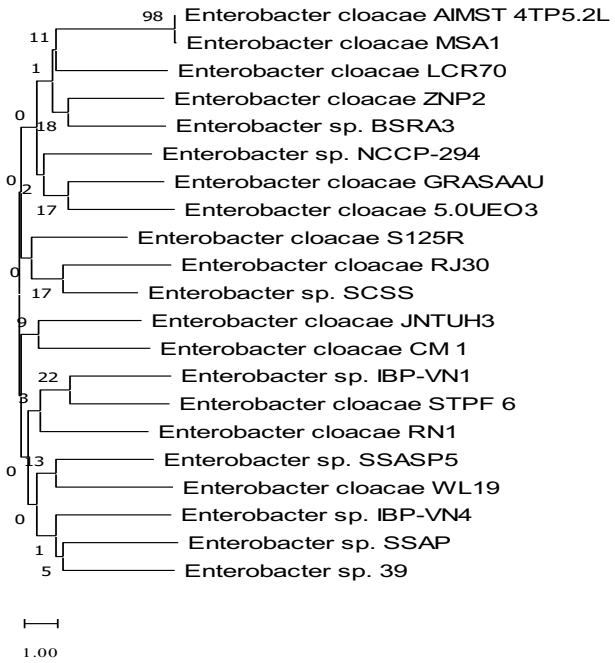

Fig. 1 The phylogenetic tree showing relationship of *E. cloacae* ZNP-2 to closely related bacteria. The NCBI GenBank database provided the 16S rRNA gene sequences of closely related species. With a bootstrap value of (n = 1000), the tree was plotted using the neighbor-joining method of software packages Mega version 6.0.

3.2 Molecular characterization of ACC deaminase

PCR amplification of the AcdS gene of strain ZNP-2 produced a 673 bp amplicon with gene-specific primers (Fig. 2A). The amplified product was confirmed by Sanger sequencing which showed a 93% identity to the AcdS sequence of Enterobacter cloacae. An accession number KM501057 was assigned to the submitted sequences. The AcdS sequence of ZNP-2 is closely related to Enterobacter

cloacae sp. and Enterobacter sp., according to gene sequence alignment and phylogenetic analysis of the AcdS gene extracted from ZNP-2 and other bacterial species (Fig. 2B).

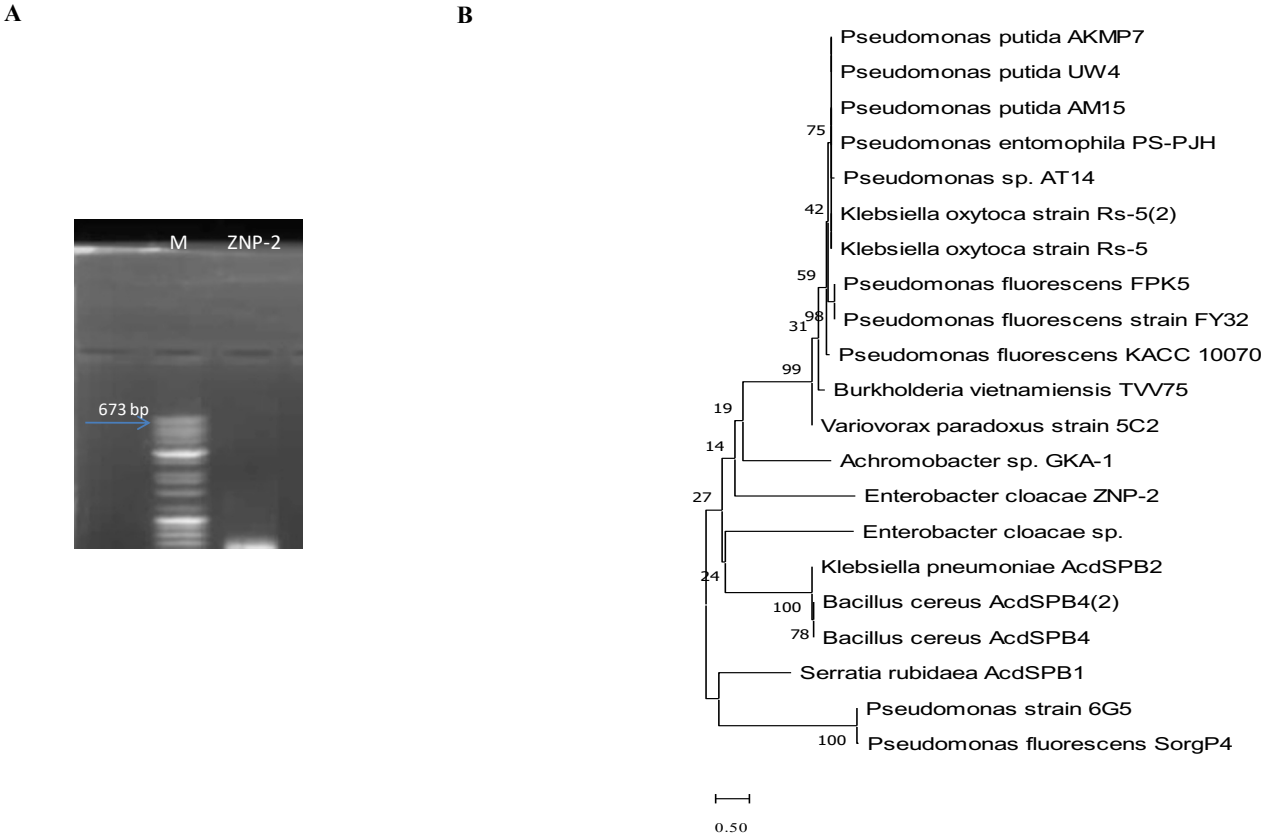

Fig. 2 AcdS gene amplification in *E. cloacae* ZNP-2 (A), dendrogram based on AcdS sequence of test isolate and other species which showed similar sequence, M: DNA ladder (B). Neighbor- joining method of software packages Mega version 6.0 was used at bootstrap value of (n= 1000).

3.3 Plant growth promoting (PGP) features

On medium supplemented with tri-calcium phosphate, an insoluble form of phosphate, strain ZNP-2 developed a clear zone surrounding its growth. Phosphate solubilization was measured and found to be 9.842±1.30 µg ml^{-1}. ZNP-2 produced 0.258±0.020 µg ml^{-1} of IAA, demonstrating positive results for phytohormone production. On N-medium, consistent growth for multiple generations showed a positive nitrogen fixation test result. The isolate tested negative for siderophore and positive for ammonia and HCN production.

3.4 Effect of isolate ZNP-2 on plant growth under salt stress

After 15 days of seed germination, data of several plant growth parameters after strain ZNP-2 inoculation were statistically examined. The inoculation of bacterial strain ZNP-2 led to a notable increase in the growth of wheat plants under salt stress conditions when compared to the corresponding un-inoculated control. Measurements of fresh weight/dry weight, photosynthetic pigments, shoot length, and root length were used to evaluate the growth. At T1, T2, and T3, salinity stress reduced shoot length by 53%, 74%, and 89%, respectively; however, ZNP-2 inoculation enhanced wheat plant growth. In comparison to uninoculated plants, ZNP-2 inoculation significantly (p=0.05) increased shoot length at treatment T-3 by 27.80%, while at treatments T1 and T-2, shoot length increased by 18.30% and 15.62%, respectively (Fig. 3A). Similarly, salt stress decreased the root length by 38%, 47% and 62% at T-1, T-2 and T-3 treatment, respectively. However, bacterial inoculation significantly improved the root length and highest increase of 31.5% was observed at treatment T-3 (Fig. 3B). Similarly, highest increase of fresh weight was 22.72% and 29.83% at treatments T-1 and T-2 in the presence of bacterial inoculation (Fig. 3C). Bacterium inoculated plants also showed improvement in dry weight at T-3 (29.90%) and T-2 (26.60%), as compared to their un-inoculated counter parts (Fig. 3D). In comparison to the corresponding control, bacterium inoculation dramatically (p=0.05) raised the chlorophyll a content of the photosynthetic pigments to 30.77% at treatment T-1. Additionally, bacterium-inoculated plants at treatments T-2 (42.19%) and T-3 (58.33%) showed a noteworthy increase in chlorophyll a

content (p=0.05) compared to uninoculated plants (Fig. 4A). Additionally, at treatment T-1, T-2, and T-3, the chlorophyll b content rose by 30.66%, 26.60%, and 29.59%, respectively, as a result of bacterium inoculation (Fig. 4B).

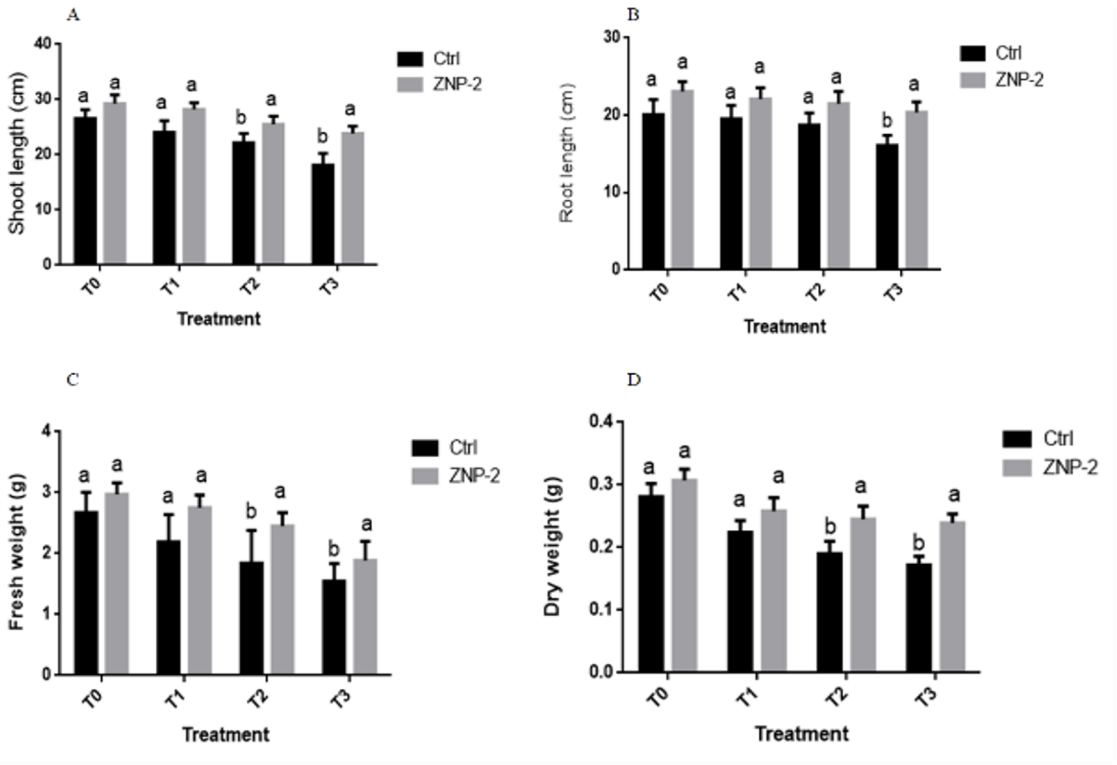

Fig. 3 Effect of strain ZNP-2 inoculation on the wheat growth under tested treatments. Different letter represents the significant difference as compared to their counterpart. Values are mean±SD of triplicate sets of five measurements (n=15).

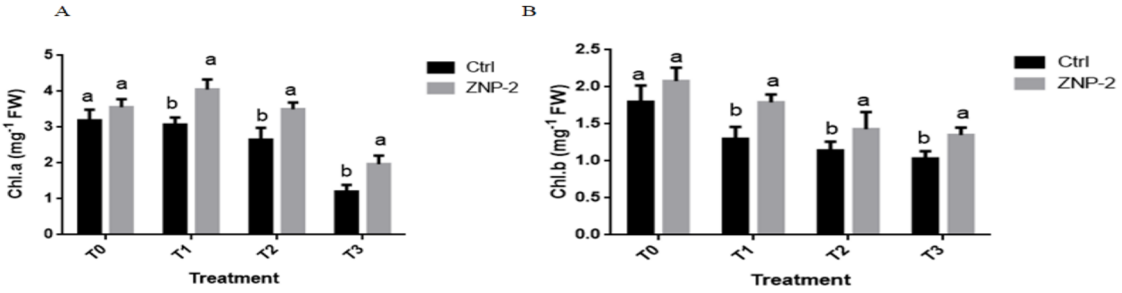

Fig. 4 Effect of inoculation of strain ZNP-2 on chlorophyll (a & b) content under tested salinity stress conditions. Values are mean±SD of triplicate sets of five measurements (n=15).

3.5 Bacterial effect on ions under salt stress

AAS was used to assess how plant growth-promoting bacteria help plants adjust to salt stress by allowing them to fine-tune the ionic balance, specifically the Na^+/K^+ ratio. Ionic analysis revealed that bacterised plants under salt stress had a decrease in Na^+ content while an increase in K^+ and Ca^{2+} content. The greatest drop in Na^+ content (54.69%) was seen at treatment T-3 when compared to the corresponding control in uninoculated control plants. When compared to their respective counterparts, the bacterium-inoculated plants at treatments T-1 and T-2 showed a significant (p=0.05) decrease in Na^+ of 25.92% and 30.57%, respectively. Treatment T-1 showed the largest significant (p=0.05) increase in K^+ (38.53%), whereas treatments T-2 and T-3 showed increases in K^+ of 30.36% and 33.68%, respectively (Fig. 5A, B). Furthermore, treatment T-1 showed a significant (p=0.05) increase in Ca^{2+} (28.33%) in comparison to other treatments, which were 19.65% and 28.18% at T-2 and T-3, respectively, in comparison to their counterparts (Fig. 5C).

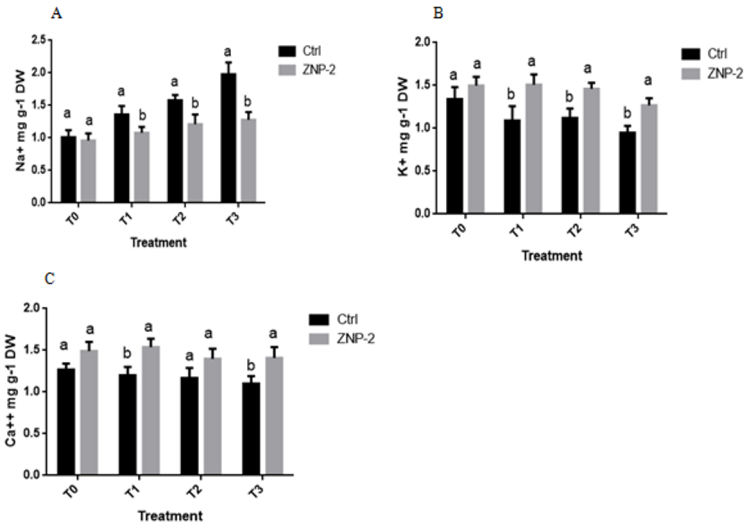

Fig. 5 Effect of inoculation of strain ZNP-2 on ionic contents under tested salinity stress. Values are mean±SD of triplicate sets of five measurements (n=15).

3.6 Biochemical analysis of plants

Proline content ranged from 70 to 155% when salinity was increased from 0 to 200 mM; however, bacterial-treated plants showed a significant decrease in proline content (p=0.05). Treatment T-3 showed the largest significant (p=0.05) reduction in proline content (41.23%), while treatments T-1 and T-2 showed decreases of 29.26% and 38.05%, respectively (Fig. 6A). Malondialdehyde (MDA) content increased as a result of rapid oxidative damage to lipids brought on by increased salinity. The MDA content of wheat plants exposed to various salt stressors was considerably reduced by bacterial inoculation. When comparing bacterial-inoculated plants to un-inoculated plants treated with corresponding salt stress, the bacterial-inoculated plants showed the largest significant (p=0.05) decrease (64.60%) at treatment T-3. In plants treated with bacteria, the MDA content decreased by 51% and 55.60% at treatments T-1 and T-2 (Fig. 6B). Although bacterium inoculation stopped the loss of TSS content, salinity also caused plants' TSS content to drop by 6-41%. When comparing bacterium-inoculated plants to their respective control plants, the treatment T-1 showed the largest significant (p=0.05) increase (67.51%), followed by T-2 and T-3 (52.54%) and (41.89%) (Fig. 6C). Similarly, at different salinity stresses, ZNP-2 inoculation also significantly (p=0.05) increased the auxin level in the range of 27% to 62% (Fig. 6D).

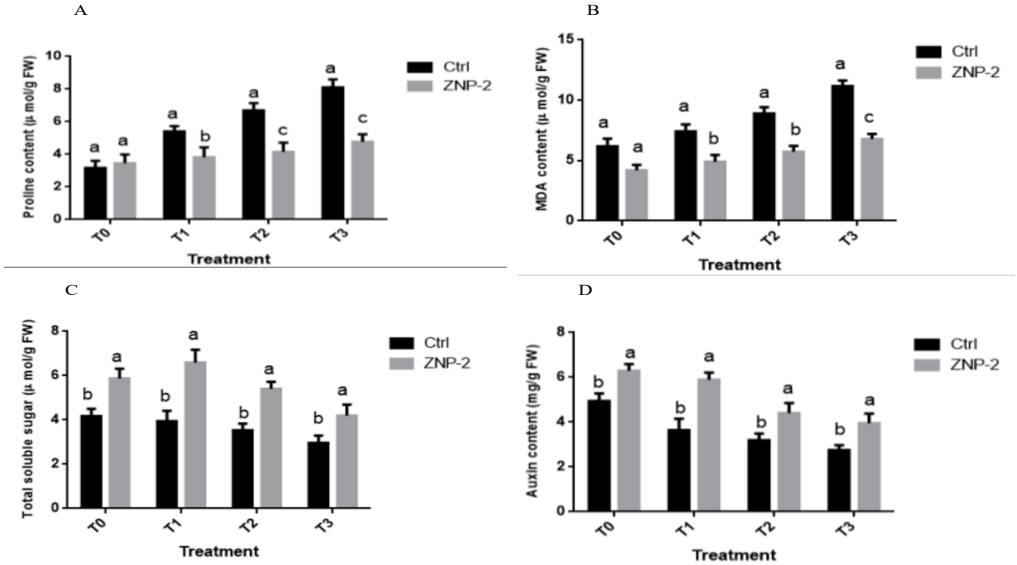

Fig. 6. Effect of ZNP-2 inoculation on proline (A) malondialdehyde (B) total soluble sugar (C) and auxin content (D) under tested salinity stress. Values are mean±SD of triplicate sets of five measurements(n=15).

3.7 Colonization

Following the experiment, the plate counting evaluation was used to assess strain ZNP-2's capacity to settle the inoculated plant root in comparison to the control. After seed germination, associative bacteria were found to be 1.1×10^3 CFU/g of root fresh weight in inoculated plants.

4. Discussion

The potential of rhizospheric bacteria isolated from *Ziziphus nullifera's* rhizosphere to promote plant growth through a polyphasic approach under gradient salt stress is reported in this study. Our investigation revealed that the bacterial isolate was an effective phosphate solubilizer and could generate IAA. Phosphate solubilization by the isolate was accompanied by decrease in pH of medium, which indicates secretion of organic acids like citric and gluconic acid in the medium (Bar-Yosef et al., 1999). Moreover, presence of phosphate-solubilizing bacteria in the soil acts as biofertilizer for sustainable plant growth. Additionally, PGPR-mediated IAA synthesis increased root elongation, which raises plant water and nutrient efficiency. Bacteria producing IAA from roots secrete more root exudates, which act as an energy source to enhance microbial growth and colonisation potential. IAA-secreting bacteria induce root elongation and root hair formation that enhances the nutrient uptake (Duca et al., 2018; Latif et al., 2016). Therefore, the IAA-production ability of isolate showed its efficacy to be used as PGPR. Many of the rhizospheric bacteria isolated [28] from different plants like wheat, maize, and rice etc. showed their ability to produce IAA [36].

Indirect PGP traits are indicated by other characteristics such as siderophore, ammonia, and HCN production. Various soil microbes produce iron chelators like siderophores which binds Fe^{3+} and provide it to the plants for better growth. Previous studies had demonstrated the production of siderophore by various rhizobacteria which enhanced the uptake of iron [37]. Similarly, the ammonia produced by microbes also benefits plants. Rhizobacteria that produce ammonia cause the soil to become alkaline (pH 9.0 to 9.5), which has a strong inhibitory effect on the growth of some fungi and nitrobacteria. Additionally, it suppresses fungal proliferation by preventing fungal spores from germinating [38]. It was discovered that the tested isolate could withstand abiotic stressors such as different pH and salinity levels. Consequently, isolate ZNP-2 may be employed as rhizobacteria that can withstand stress in soil that is subject to environmental stressors. According to phylogenetic evolutionary analysis and the BLAST search, isolate ZNP-2 is a member of the Enterobacter sp. Previous studies had showed the ACCD-producing rhizobacteria belonging to Enterobacter species for their potential to improve the plant growth under abiotic stress conditions. Moreover, ACCD-producing strains belonging to Burkholderia sp., Pseudomonas sp., Klebsiella sp., and Bacillus sp. showed their potential for salt tolerance in wheat, rice, maize by minimizing the ethylene production under stress. The prominent role of ACCD was further confirmed by mutation of AcdS gene in Enterobacter cloacae and *Burkholderia phytofirmans*, which leads to decrease of plant growth promotion in their host plant. Jaemsaeng et al. observed the growth of rice under salinity stress following inoculation of ACCD-producing Streptomyces sp. GMKU 336 by reduction of ethylene. In the present study, ZNP-2 showed both ACCD and IAA production. The ACCD production decreases the ethylene level, while IAA stimulates plant growth. Thus, the synergistic effects of IAA and ACCD secretion by ZNP-2 are important for enhancing plant tolerance to salinity stress.

Salinity stress leads to accumulation of salt ions and also affects the ionic homeostasis which severely affects the plant growth.. Previous finding suggested that maintaining a higher K^+/Na^+ ratio is favourable for salt tolerance in plants [39]. In this study, salt-stressed wheat plants without bacterial inoculum showed a gradual decrease in K^+ content while a significant increase in salinity-induced Na^+ content. However, wheat plants inoculated with strain ZNP-2 showed significantly reduced Na^+ content and enhanced K^+ content. The results were in agreement with previous study where ACCD-producing Dietzianatrono limnaea and Streptomyces GMKU 336 protected the plants from salinity-induced damages via enhancing the up-regulation of Na^+/H^+ antiporter gene (NHX1), involved in maintaining the homeostasis of Na^+ in the cytoplasm. Similarly, cotton plant inoculated with ACCD-producing Klebsiella showed higher K^+ content that resulted in salt stress protection. In the current study, we found that all salinity-treated uninoculated wheat plants showed a decrease in Ca^{2+} levels as a result of the imposed salinity stress. However, the Ca^{2+} content of plants inoculated with strain ZNP-2 remained higher. Our results are in agreement with previous findings where plant growth promoting bacterium Pseudomonas increased the Ca^{2+} content in eggplant and cotton plant. This increase in Ca^{2+} content could be due to upregulation of Cam 1-1 gene, whose expression plays a significant role in binding of Ca^{2+} to the calmodulin complex and also Ca^{2+} signal transduction network.

Besides, compatible solutes or osmolytes also play a major role in providing the osmotic adjustment under stress conditions.. The current study found that ZNP-2-inoculated plants had lower proline content than uninoculated plants, indicating that bacterial-inoculated plants under salt stress experienced less severe stress. Soil salinity also increased ROS production and it finally resulted in increased membrane lipid peroxidation [40]. Bacterial-inoculated plants showed significantly decreased MDA content which illustrated the stress-protective effect of bacterial inoculation. The presence of total soluble sugars also maintains the proper osmotic balance in plants under salt stress. Similarly, improvement in auxin levels was observed in ZNP-2-inoculated plants growing with or without NaCl stress [40]. Our results are in agreement to previous study, where ACCD and IAA-producing bacteria *Leclerciaade carboxylata* significantly improved the growth of tomato plants under salinity stress.

Conclusion

ACCD-producing rhizosphere bacterium *Enterobacter cloacae* ZNP-2 isolated from the rhizosphere of *Ziziphus nullifera* grown in the desert region, was able to produce IAA and ammonia, and solubilize phosphate. *Enterobacter cloacae* ZNP-2 significantly improved the growth of wheat plants by alleviating the different levels of salinity. The test isolate also regulates the ion transporters thereby favouring the K^+/Na^+ ratio. The application of bacterium induced the production of certain osmolytes to minimize the salinity-induced oxidative damages. The obtained results illustrated that the bacterium with ACCD activity to minimize the stress-induced ethylene level can be used to facilitate plant growth under abiotic stressors. The observed results of the present study suggest that PGPR can play a vital role in conferring salt tolerance in plants. However, further work is required to evaluate the efficiency of the isolate under actual field conditions to confirm the test isolate as an effective bio-inoculant for mitigation of the negative effect of salt stress on wheat plants.

Credit authorship contribution statement

All authors have reviewed, approved, and contributed in accordance with the order of authorship as presented in the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

The authors acknowledge the infrastructure support of Department of Biotechnology, JIIT Noida.

Financial support

There has been no financial support from any funding agency for the research work.

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