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ORIGINAL PAPER

Prolific contribution of *Pseudomonas protegens* in Zn biofortification of wheat by modulating multifaceted physiological response under saline and non-saline conditions

Jyoti Singh[1] · Ajay Veer Singh[1] · Viabhav Kumar Upadhayay[1] · Amir Khan[1] · Ramesh Chandra[2]

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Abstract

The current study aimed to characterize the contribution of bacterium CP17 in zinc (Zn) biofortification in wheat under saline and non-saline conditions. This bacterial strain effectively solubilized Zn and tolerated up to 20% NaCl concentration. The Zn-solubilization potential was also quantified using AAS in a liquid broth supplemented with zinc oxide and zinc carbonate at various NaCl concentrations. Lowering the pH of liquid broth and analyzing a wide range of organic acids (thioacetic acid, glutamic acid, carboxylic acid, propionic acid, and so on) using UPLC-MS provided mechanistic insight for zinc solubilization. This strain was also shown to possess plant probiotic characteristics like phosphate solubilization, production of siderophore, indole acetic acid (IAA), exopolysaccharide (EPS), ACC deaminase, and ammonia. CP17 was identified as a *Pseudomonas protegens* based on the 16S rRNA gene analysis. In addition, the amplified product of the ACC deaminase producing gene (acdS) provided a molecular indication of the strain's endurance towards stress. The towel paper assay confirmed that the inoculation of *Pseudomonas protegens* CP17 significantly increased wheat seedlings' germination, growth, and biomass under different NaCl concentrations (0 mM, 100 mM, and 150 mM). Afterward, In situ pot experiment study was designed with the inoculation of *Pseudomonas protegens* in wheat under saline and non-saline conditions. The harvested wheat plants showed an elevated pattern of zinc content in the grain (i.e. 24.33 and 29.33mg/kg), straw (i.e. 45.73 and 50.23mg/kg) and soil (i.e. 0.978 and 1.32mg/kg) under saline and non-saline conditions, respectively and shown significant improvement over control. The results of the pot study revealed the amelioration in plant health, yield and uptake of available zinc from rhizospheric soil to straw and grain, along with enhanced dehydrogenase and phosphatase activities of rhizospheric soil under saline and non-saline conditions. This study supports the integrative role of *Pseudomonas protegens* CP17 as a bioinoculant for the efficacious strategy of zinc biofortification and growth promotion in wheat and ensures sustainable nutrient quality production under salinity stress.

Keywords Atomic absorption spectrophotometer · ACC deaminase · Biofortification · PGPR · Salinity · UPLC-MS

Introduction

The exponential rise in the world's population presents a vital challenge for existing agricultural practices to provide a promising solution for the steadily increasing demand for food production. Increasing conspicuous demand for food production is analogous to loss of arable land due to mounting severity of soil obliteration and environmental conditions (Bharti et al. 2016; Cole et al. 2018). Salinity is one among various foremost abiotic stresses which enormously reduces the yield and productivity by limiting the crop growth in barren and semi barren regions globally. The severity of escalating soil salinity will likely be intensified

✉ Ajay Veer Singh
ajaygbpuat@gmail.com

[1] Department of Microbiology, College of Basic Sciences and Humanities, Govind Ballabh Pant University of Agriculture and Technology, 263145 Pantnagar (U. S. Nagar), Uttarakhand, India

[2] Department of Soil Science, College of Agriculture, Govind Ballabh Pant University of Agriculture and Technology, 263145 Pantnagar (U. S. Nagar), Uttarakhand, India

with increased food demand and loss of prime agricultural land (Kearl et al. 2019). Over the last few decades, approximately 10million hectares of agricultural land have been destroyed due to soil salinization. It is estimated that by 2050, approximately 50% of the arable land will be affected through salinity worldwide (Machado and Serralheiro 2017; Iqbal et al. 2018). High level of soil salinization limits the plant's capability to acquire water. If the salt concentration is too high in the soil, water may flow backward from plant roots to the soil. This situation creates osmotic deficit conditions of salinity and ionic toxicity, which adversely affect the plant system in terms of growth, productivity and yield (She et al. 2018). Excessive concentration of ions (Na, Cl, K, Mg, Ca) in soils disturb the integrity of the plant system and alter several enzymatic as well as metabolic processes, which may cause nutritional imbalance, metabolic dysfunctioning, ion toxicity induced metabolism, reactive oxygen species accumulation and subsequently affect cell division (Darko et al. 2017; Singh et al. 2020). These effects of salinity also decrease photosynthetic capacity, limit the germination rate, protein synthesis, energy production, seedling growth and even cause the death of the plant (Jiang et al. 2017; Iqbal et al. 2018).

Despite these factors, salt stress can also direct micronutrient deficiency in the soils due to imbalances or competition of Na^+ and Cl^- with other micro and macro elements (Hu and Schmidhalter 2005). Among micronutrients, zinc (Zn) is an extremely vital nutrient requisite throughout the life cycle of each cereal crop. It is classically the second most copious micronutrient in organisms after iron and is mostly present in each class of the enzyme (Bapiri et al. 2012). The vital information about Zn dynamics in soil, water and plants is an imperative step for accomplishing sustainable solutions to Zn deficiency problems in plants and humans. Zn is crucial for some biochemical progressions such as nucleotide and cytochrome synthesis, enzyme activation, membrane activity maintenance and chlorophyll production (Khan and Singh, 2021). The prime reason for Zn deficiency in humans and plants may emerge from poor diet and barren soils, respectively. Aproximately 165million childrens suffering from stunted growth have a risk of low physical capability and cognitive development (Sunithakumari et al. 2016). Deficiency of Zn is also related with acute respiratory infections and diarrheal diseases, which are paramount causes of infant mortality. Similarly, in plants, Zn insufficiency directs leaf chlorosis, old leaf necrosis, reduced biomass and photosynthesis, and reactive oxygen species accretion in most of the cereal crops (Wang et al. 2019).

Triticum aestivum (Wheat) is classified among the most widely cultivated cereal grain worldwide. It is a chief source of protein, vitamins, dietary fibers with multiple essential nutrients (Singh et al. 2017). Approximately 60% of wheat productivity, nutrient quality and wheat crop yield are lost by various environmental stresses in which salinization is a chief factor (Vimal et al. 2018). Consequently, for conservation of food security and biodiversity under saline conditions required changes in policies, techniques and practices of wheat cultivation are necessary. World food security can only be achieved through the development of eco-friendly methods and green technologies for sustainable agriculture and the development of salinity tolerant food crops (Imadi et al. 2016). Among various determined efforts to deal with this problem, the implementation of microbial technology in saline soil conditions is desired strategies for development of micronutrient dense staple food crops. It is an eco-friendly and effective approach for present and future, which can enhance the soil fertility, yield and nutrient quality in crops under salinity stress for sustainable agriculture (Jha et al. 2012).

Rhizobacteria is a term used to symbolize a bacterial community capable of colonizing the rhizosphere, which leads to stimulation of growth and defense of plants in various biotic and abiotic stresses environment (Khan et al. 2020a). Plant growth-promoting mechanisms of these rhizobacteria may include phosphate solubilization, siderophore production, and Zn solubilization, biological nitrogen fixation, 1-aminocyclopropane-1- carboxylate (ACC) deaminase production, phytohormone production, antagonistic activity, bioactive volatile organic compounds production, and induced systemic resistance (Mitra et al. 2018; Khan et al. 2020b). The potentiality of rhizobacteria in the agricultural system is progressively increasing as it provides a unique approach to alleviate abiotic stress by balancing the nutrition in crop systems. Voluminous literature has already been published revealing that alkaline, sodic, as well as saline soils often account for Zn unavailability in the soil system. Moreover, the unavailability of Zinc in soil leads to its deficiency in plant system (Shen et al. 2011; Dinesh et al. 2018). However, rhizobacteria have shown good ability to ameliorate Zn accessibility in different plant regions (Whiting et al. 2001; Fasim et al. 2002; Biari et al. 2008; Subramanian et al. 2009; Khan et al. 2019). The rhizosphere microflora may cause Zn translocation or solubilization through wide arrays of mechanisms involved in nutrient solubilization, including organic acid secretion, production of chelating agent, protons, and oxido-reductive systems on cell membranes, which have been reported in soil microflora. Organic acids produced by rhizosphere microflora show a downward trend in pH in the nearby soil, thus creating a suitable environment for solubilizing insoluble forms of nutrients (such as phosphate, zinc, iron, and potassium). Other mechanisms include the secretion of iron chelating agents, "siderophores," which are believed to play a key

role in solubilizing micronutrients, such as iron and zinc. These solubilized nutrients can efficiently uptake by plants (Zheng et al. 2018; Upadhayay et al., 2021).

It is a well known fact that a particular bacterium prefers to grow in a specific environment. Therefore, exploration of new isolates is always required and it is also essential to aware with the mechanisms involved for biofortification by these salt resistant rhizobacteria. To date, there are no reports available on the function of PGPR for Zn biofortification via amelioration of salt stress in wheat cultivated under saline impacted soils. In consideration to these facts, the objective of current study is to elucidate the microbial approach for development of Zn dense staple wheat crop via enhancing soil fertility and plant health for conquering malnutrition under saline conditions. In addition, the present study involves the isolation of salt tolerant rhizobacteria bearing massive plant growth promoting traits. Here, we focused on different mechanistic insights involved in Zn solubilization and mitigation of salt stress by bacterial isolate and further evaluated its action for growth and Zn assimilation in wheat grain under saline conditions.

Materials and methods

Sample collection and bacterial isolation from rice rhizosphere

A geographically salt-affected agricultural site (pH 10.2 ± 2, EC 6.61 dSm^{-1}) of rice located in Chiplun, Maharashtra (17°53'0" N, 73°52' 0" E), India was selected for bacterial isolation. Soil surrounding the rice root (rhizospheric soil) was collected and stored in sterile polyethylene zip bags and transported to laboratory under sterile conditions and kept in refrigerator till further use. For bacterial isolation, 1g of rhizospheric soil sample was dissolved in 10mL of distilled water (dH_2O) and the soil filtrate was successively diluted up to 10^{-7} and grown on Nutrient agar medium for 24h at 28 ± 2°C. The bacterium was subcultured and purified by following the standard protocol (Shakeel et al. 2015). The purified cultures were retain in Nutrient agar slants at 4°C and in glycerol stock at -80°C.

Screening of zinc solubilizing bacteria

The potentiality of bacterial isolates for Zn solubilization was tested on Minimal agar amended with 0.1% ZnO and $ZnCO_3$. 5 µL of each active bacterial culture having absorbance stabilized to 0.5 was inoculated on sterile Minimal agar plates containing 0.1% of respective Zn content. Plates were kept in incubation for 36 to 96h at 28 ± 2°C. The emergence of halo zone on the media plate signifies their potential of Zn solubilization. The experiment was carried out with replicates. The Zn solubilization efficiency of bacterial isolates were obtained by following formula described by Mumtaz et al. (2017):

Solubilization efficiency % (S.E.) = (diameter of halo zone / diameter of colony) × 100.

Determination of optimum tolerance level

The bacterium was inoculated in nutrient broth medium containing different NaCl concentrations (5%, 10%, 15%, 20% w/v) and incubated at 28 ± 2°C for 7 days. The growth of bacterium was scrutinized by evaluating the absorbance at 600nm using UV-Visible spectrophotometer (Thombre et al. 2016). The experiment was carried out with replicates.

Plant growth promonting traits of bacterial isolate

Siderophore production

Siderophore production was determined through Chrome azurol S (CAS) test (Schwyn and Neilands 1987). Log phase bacterial culture was spot inoculated on sterile Petri plate containing nutrient agar medium supplemented with CAS dye. Plates were kept on incubation at 28 ± 2°C for 48 to 72h and were observed for orange or yellow halo zones around bacterial colony.

Production of siderophore was quantitatively measured by inoculating bacterial isolate in Minimal broth medium. The tubes were incubated at 28 ± 2°C for 72h and shaking at 120rpm. Afterwards, the incubated medium centrifuged at 10,000rpm for 15min and 0.5 mL of culture supernatant was blend with 10µL of shuttling solution (5- sulfo salicylic acid) and 500µL of CAS reagent. The optical density was find by spectrophotometer at 630nm against a reference solution containing uninoculated minimal broth medium with 500µL CAS reagent and 10µL shuttling solution. The % siderophore content was measured by following formula:

% Siderophore units = $[(A_r - A_s)/ A_r] \times 100$.

Where, Ar = absorbance of reference; As = absorbance of the sample.

Phosphate solubilization

The estimation of phosphorus solubilization was analyzed according to Premono et al. (1996). The sterilized Pikovaskya medium poured in sterile petri plates. A loopful bacterial cultures were inoculated in sterile Pikovaskya medium

and incubated for 72h at 28 ± 2°C. A clear halo zone indicates their phosphate solubilizing efficiency.

Moreover, quantification of phosphorous solubilization was done NBRIP-BPB broth according to the protocol of Nautiyal et al. (2003). Bacterial isolate was inoculated in 25 mL of NBRIP-BPB medium and incubated for 72h at 28 ± 2°C with constant shaking on at 120rpm. The bacterial cultures were centrifuged at 6000rpm for 5min and 1mL of culture supernatant was collected. Afterwards, 0.4 mL of 10% TCA, 0.4 mL molybdate solution, 0.2 mL of color reagent and 4 mL triple distilled water were added and incubated at room temperature for 5min for blue color development. For estimating the quantified amount of solubilized phosphorus, a standard curve of KH_2PO_4 was prepared by maintaining 1mg mL^{-1} as stock solution, and the absorbance was obtained at λ 640nm by using spectrophotometer (Fiske and Subbarow 1925).

IAA production

Indole acetic acid (IAA) production was examined by the colorimetric method illustrated by Patten and Glick (2002). Bacterial isolate was inoculated in 5mL sterilized Luria broth medium supplemented with filtered sterilized L- tryptophan (50µg mL^{-1}). Culture broth medium was incubated for 2 days at 28 ± 2°C. Afterwards, the medium was centrifuged on 5,500rpm for 10min and pellet was discarded. An aliquot of 1mL supernatant was vigorously mixed with 4 mL Salkowski's reagent and incubated for 20min at room temperature. The absorbance was determined after the development of reddish-pink color at 540nm through a spectrophotometer. Production of IAA by bacterial isolates was resolute by preparing the standard curve of IAA using different concentrations viz. 0.2, 0.4, 0.6, 0.8, 1mL.

HCN production

Sterile nutrient agar amended with glycine was prepared to assess HCN production as per procedure described by Donate-Correa et al. (2005). A log phased bacterial cultures were streak on glysine amended Nutrient agar (4.4g/L). The sterile filter paper strip soaked within picric acid solution was placed underside of lid Petri plate and sealed by parafilm. Plates were incubated for 72h at 28 ± 2°C. A color change of filter paper strips from yellow to orange or brick red signified the positive results.

Exo-polysaccharide production

Estimation of EPS production by bacterial isolate was determined according to Siddikee et al. (2011). An aliquot of 20 µL from overnight grown active culture (OD = 0.40) was inoculated in 50 mL Basal medium. The medium was incubated at 28 ± 2°C for 3 days at 120rpm in a rotary shaker. After incubation, medium suspension was spin at 10,000rpm for 10min. The EPS was then precipitated by addition of two volumes of ice-cold isopropanol into pellet and incubated overnight at 4°C. Later, the centrifugation process was again done at 7,000rpm for 20min and pellet was collected. Collected pellets were dried at 60°C. The dry weight of pellet was recorded to resolve the exo-polysaccharide produced by bacterial isolate.

ACC deaminase activity

The ACC deaminase enzyme amplitude was detected by procedure illustrated by Penrose and Glick (2003). Dworkin and Foster (DF) minimal salt medium supplemented with ACC as exclusive nitrogen source was used to estimate ACC deaminase activity of bacterial isolate. A mid log phased bacterial culture was spun at 8,000rpm for 10min at 4°C. Pellet was kept and washed with DF salt minimal medium followed by additional centrifugation at 8,000rpm for 10min at 4°C. further pellet was suspend in 7.5mL of DF salt minimal medium supplemented with 3mM ACC and incubated overnight at 28 ± 2°C with continuous shaking (at 120rpm) on a rotary shaker. Afterwards, the centrifugation was done again at 8,000rpm for 10min at 4°C. Further supernatant was remove and cell pellet was washed with 5mL of 0.1M tris HCl (pH 7.6) and centrifuged at 16,000rpm for 5min at 4°C. Pellet was collected and suspended in 600µL of 0.1M tris HCl (pH 8.5). 30µL of toluene was added in cell suspension followed by vortexing the suspension for 30s. 100µL of tolunized cell suspension was kept aside and used for protein measurement by adding 20µL of 0.5M ACC followed by vortexing and then incubating for 15min at 30°C. After incubation, 1000µL of 0.5M HCl was supplemented into the suspension and spun at 16,000rpm for 5min at room temperature. 1 mL supernatant was transferred into 800µL 0.56M HCl and vortexed. Further, 800µL of 2, 4 dinitrophenyl hydrazine (DNPH) reagent (0.2% DNPH in 2M HCL) was added in the sample, vigorously mixed and incubated for 30min at 30°C. Afterward, 2mL aliquots of 2N NaOH was mixed and absorbance was taken at 540nm. 200µL aliquots of 0.1M tris HCl (pH 8.5) with supplementation of 20µL of 3mM ACC was taken as positive reference and 200µL aliquots of tolunized cells without supplementation of ACC was taken as negative reference.

Ammonia production

Ammonia production efficiency of bacterial isolate was checked according to the method of Cappuccino and Sherman (1992). The culture was incubated in 10 mL Peptone

broth at their optimum temperature i.e. 28 ± 2°C for 48h with continuous shaking at 120rpm in a rotary shaker. Afterward, 500µL of Nessler's reagent was added to each test tube of bacterial culture and incubated for 10–15min at room temperature. Development of yellow color represents positive results.

Quantitative determination of zinc solubilization in different range of NaCl concentration

An aliquot of 100µL active culture was implicated in 50 mL Minimal medium supplemented with 0mM, 100mM, 200mM, and 300mM (w/v) NaCl and incubated for 72h at 28 ± 2°C in a rotary shaker at 120rpm. The medium treated with bacterial samples as well as uninoculated control was taken out after 3 days and spun for 15min at 10,000rpm to eliminate the cellular debris. The pellet was discarded and supernatant was kept and filtered by using 2µm membrane filter. The supernatant filtrate was used to determine the solubilized Zn content at 213.90nm through atomic absorption spectrophotometer (Sunithakumari et al. 2016).

Estimation of organic acids

The organic acids, responsible for Zn solubilization were evaluated according to Costerousse et al. (2017). In brief, 50 mL autoclaved minimal medium containing ZnO was transferred in 100 mL flasks and subsequently inoculated with CP17 strain. The experiment was performed for 9 days at 28 ± 2°C in a rotary shaker at 120rpm. 20 mL aliquots were taken out from the flasks at 1st, 2nd, 4th, 6th and 9th days of incubation. Afterward, the sample was spun at 2,500rpm for 10min, and the supernatant was filtered via millipore filter (0.22µm pore size). The culture filtrate of solubilized ZnO was measured for changes in pH pattern at each point of time through pH meter. Furthermore, the 4-day old filtrate was analyzed for organic acids through ultra-performance liquid chromatography (UPLC) integrated with a mass spectrometer (MS). UPLC-MS was executed at the advanced instrumentation research facility (AIRF), JNU, New Delhi, India. An ethylene-bridged hybrid amide column (Acquity UPLC, column internal diameter, 200µm; column length, 15cm; particle size, 1.7µm) was used to separate organic acids. Moreover, the obtained results were further used for identification of respective organic acids by using METLIN database.

16SrRNA gene sequencing and phylogenetic identification

Extraction of bacterial genomic DNA was done with standard procedures outlined by Bazzicalupo and Fani (1996). The extracted genomic DNA was subjected to PCR amplification of 16S rDNA by using eubacterial universal primer set 27F (5' AGAAGTTTGATCCTGGCTCAG 3') and 1492R (5' TACCTTGTTACGACTT 3'). 16S rRNA gene for bacterial isolate was amplified in 25 µL reaction mixture containing PCR Buffer 2.5 µL; $MgCl_2$ 2 µL; 2 mM dNTPs 1 µL; Primers 0.5 µL each; Taq DNA polymerase 0.5 µL; Template DNA 2 µL; Sterile deionized water 16 µL. Initial denaturation was provided at 95°C for 7min following 35 cycles of 94°C for 1min, 52°C for 1min, 72°C for 1min and final extension at 72°C for 10min (Kamran et al. 2017). The amplicon was separated through electrophoresis by applying 2µL of loading dye (1X) with 4µL of amplified DNA product into well of 1.5% agarose gel containing ethidium bromide (0.5µg mL^{-1}). The sequencing was executed from Chromus Biotech Pvt. Ltd. Banglore, India by using ABI 3500 genetic analyzers. The 16S rDNA sequence's electrophenogram data was authenticated through Chromas 2.33 software. For phylogenetic analysis, 16S rDNA sequences of CP17 and reference sequence retrieved from the NCBI GenBank database were aligned with the Clustal W algorithm. The consequential alignments were assessed with MEGA 5.0 for phylogenetic tree construction by inferring it with the neighbor-joining method.

Amplification of *acdS* gene

To detect the gene coding for an enzyme ACC deaminase, bacterial genomic DNA extraction was done and *acdS* gene was amplified with the two sets of degenerative primers. Primers F1936 (forward) 5' GH GAM GAC TGC AAY WSY GGC 3' F1937 (forward) 5'MGV AAG CTC GAA TAY MTB RT 3' F1938 (reverse) 5'AT CAT VCC VTG CAT BGA YTT 3' F1939 (reverse) 5' GA RGC RTC GAY VCC RAT CAC 3' (Blaha et al. 2006). Standard PCR reagents and thermal cycles were utilized according to Blaha et al. (2006). Amplification was done on thermal cycler, PTC-200 thermal cycler (M.J. Research). The amplicon was separated by agarose gel electrophoresis on 1.5% TAE buffer with ethidium bromide staining (0.5µg mL^{-1}) to visualize the amplified product on the gel documentation system Gel-DocMega (Biosystematica).

Effect of bacteria on wheat germination and growth-promoting traits under salinity stress

The towel paper assay was done to examine the bacterial effect on wheat seedlings germination under saline conditions, as per the method described by (Parveen et al. 2018) with some modifications. To carry out the assay, *Triticum aestivum* (Wheat) var. PBW 373 seeds were used and obtained from Norman E. Borlogue Crop Research Center, GBPUA&T, Pantnagar, India. The surface sterilization of seeds was done with 95% C_2H_5OH for 30s and followed by 0.1% $HgCl_2$ for 3min. For removing the toxic effects of $HgCl_2$, the seeds were washed 8–10 times by autoclaved dH_2O (Etesami et al. 2009). Subsequently, bacterization of 100 surface-sterilized seeds was done at 1.9×10^8 CFU $seed^{-1}$ after inoculating with *Pseudomonas protegens* CP17 for 4h at ambient conditions. The inoculated seeds were placed in between sterile and moistened towel paper. Towel paper was kept in a tilted position in the seed germinator at 20°C for 8 days. Towel paper was sprayed with sterile distilled water supplemented with 0mM, 100mM and 150mM (w/v) NaCl and analyzed for germination on a daily basis.

In situ plant growth promotion

In situ plant growth promotion study was conducted in plastic pots (25cm diameter) under greenhouse with wheat var. PBW 373. The experiment was executed in duration of November 2017 to April 2018. For seed bacterization, wheat seeds were inoculated in overnight grown bacterial culture suspension (10^8 CFU/mL). The bacterization was done for 4h in a rotary incubator shaker at 120rpm. Uninocualted seeds were taken as control. To compare the effectiveness of bacterial strain for growth promotion and biofortification of wheat a pot trial was set up in 2 sets, first for non stressed soil from Pantnagar, Uttarakhand, India (sandy loam) having pH 6.91 and EC 0.40 dS/m, 3.66mg/kg available iron, 0.83mg/kg available Zn and 18.7mg/kg available P and second for stressed saline soil (silty clay loam) from village Mehrara (Uttar Pradesh,India) having pH 8.41, EC 4.96 dSm-1, 2.29mg/kg available iron, 0.21mg/kg available Zn and 10.7mg/kg available P. The experiments were performed in CRD (Completely randomized design) with three replications. The biochemical and physiological parameters of plant were analyzed at 45 and 75 days after sowing (DAS). Moreover, the agronomical parameters were recorded after the harvesting of plants.

Measurement of total photosynthetic pigments

Carotenoid and chlorophyll content were measured in wheat leaves at 45 and 75 DAS. The chlorophyll and carotenoid were determined according to Hiscox and Israelstam (1979) protocol.

Total proline content

Fresh leaves were used to measure the total proline content as per Bates et al. (1973). In brief, 100mg fresh leaves of wheat were mixed with 3% sulphosalicylic acid (1.5 mL) and spun for 10min at 10,000rpm at 4°C. 2mL acid ninhydrin + 2mL glacial acetic acid was reacted with supernatant (100 µL) for 60min at 100°C. The reaction was terminated by ice incubation. Afterward, 1 mL toluene was used to extract the proline and absorbance was evaluated at 520nm. Total proline was predicted by standard curve of proline.

Lipid peroxidation

Quantity of lipid peroxidation was determined as per the procedure of Heath and Packer (1968) via estimating malondialdehyde level produced by thiobarbituric acid (TBA) reaction. 0.1g of fresh leaves were homogenized by adding 1mL of 0.1% (w/v) TCA and centrifuged for 10min at 15,000rpm. 0.5mL of supernatant was added with 1.5 mL of 0.5% TBA and diluted in 20% TCA. The reaction was incubated at 9°C for 25min and terminated by incubating the mixture on ice. Optical density was recorded at 532 and 600nm. The difference between specific absorbance of 532 and 600nm was taken for measurement of MDA content. MDA quantity was deliberated through their molar extinction cofficient (155 mM^{-1cm-1}).

Catalase activity

Catalase activity of plant samples was measured by following the procedure of Luck (1965). In brief, 0.5g fresh leaf tissues were mixed in 10mL 0.067M phosphate buffer (pH 7). The sample was spun for 10min at 8000rpm. Afterwards, supernatant was kept for enzyme assay.100µL of the enzymatic extract was then reacted with 3 mL H2O2 phosphate buffer (pH 7), and mixed thoroughly. Time required for decline the optical density by 0.05 units was logged at 240nm by using a spectrophotometer. The solution containing only H2O2 phosphate buffer was taken as control.

Electrolytic leakage

The electrolytic leakage in leaf tissue was calculated as per the procedure illustrated by Dionisio-sese and Tobita (1998). The fully developed leaves were excised, chopped in small discs from each treatment and washed 2–3 times with dH2O to clean and eliminate the surface adhered ions. The clean chopped leaves were then placed in closed vials containing 10mL of dH_2O and incubated at ambient temperature (25 ± 2°C) for 1day at 100rpm on a rotary shaker. Afterward, the leaf sample solution was deliberated to calculate electrical conductivity (C1). Samples were then autoclaved at 121°C for 20min at 15psi (100kPa) atmospheric pressure and electrical conductivity was again measured (C2) after lowering down the solution temperature. Electrolytic leakage was calculated by subsequent formula:

$$Electrolytic\ leakage(\%) = (C1.C2) \times 100$$

Relative water content (RWC)

Relative water content of leaves was measured at each sampling at various intervals by the method illustrated by Weatherley (1950). The fresh weight of the leaf sample was weighed and leaves were allowed to dip in dH_2O for at least 3 to 4h. Afterward, the fully turgid leaves were observed and weighed. Subsequently, the turgid leaves were desiccated at 70°C for 72h inside the hot air oven and further, the weight was re-recorded.

Assessment of micronutrient content in rhizospheric soil and plant

Rhizospheric soil treated with *P. protegens* CP17 was analyzed using DTPA extraction process (Lindsay and Norvell 1978). 10g dried soil was weighed and poured into 100 mL flasks. In this suspension, DTPA extract (20mL) was added and kept for 2h at 25°C in rotary shaker at 120rpm. Subsequently, the soil suspension was filtered by filter paper (Whatman no. 42) and transferred to storage vials. Then the filtered suspension was taken for analyzing Zn content using AAS.

However, the Zn content in wheat straw and grain was estimated through the procedure of Singh et al. (2011). 0.1g of seed samples and 0.5g of straw samples were weighed and transferred into the digestion bottles/tubes, separately. In each digestion tube 10 mL mixture of nitric acid: sulfuric acid: perchloric acid (10: 1: 4 v/v/v) was added. These digestion tubes were put on a hot plate for complete digestion. After complete digestion, the digested solution was cooled down and 5 mL of 6N HCL was added and volume made up to 50mL with distilled water. The filter paper (Whatman no.1) was utilized to filter the digest and transferred to storage vials. Then finally filtered samples were used for analyzing Zn content through AAS. The AAS was calibrated by using Zn standard with 50, 100, 150 and 200ppm concentrations for radiations and measure the absorbance to calculate the correlation coefficient by the instrument.

Soil enzymatic activity

Soil dehydrogenase activity (DHA)

The DHA activity of soil was evaluated for comparative estimation of biological activity under saline and non saline soil. It is measured by the reduction of 2,3,5 triphenyl tetrazolium chloride into red-colored triphenyl formazan. Soil DHA activity was measured by following the procedure described by Casida et al. (1964). In brief, 5g soil sample was prepared with 0.25g $CaCO_3$ and dissolved in 5mL 2% TTC solution (w/v). However, the soil taken as control was treated only with 5mL of distilled water. The soil solution was incubated at 37°C for 8h in room temperature at 120rpm. Afterward, to extract the reduced triphenyl formazan, 25mL of acetone was added and centrifuged at 4500rpm for 10min at 4°C. Whatman no. 1 filter paper was utilized to filter the suspension and absorbance was assayed in a spectrophotometer at 485nm. The activity of dehydrogenase enzyme was expressed in µg formazan/g soil/day.

Soil phosphatase activity

The phosphatase activity was measured to determine the reaction rate of phosphatase in saline and non saline soil, with possible consequences of nutrient cycling. The phosphatase activity was calculated according to the procedure illustrated by Tabatabai and Bremner (1969) using a modified universal buffer (MUB) Skujins et al. (1962). 1g soil was treated with 0.25mL of toluene and 4mL of modified universal buffer (pH 11 for alkaline phosphatase and pH 6.5 for acid phosphatase) followed by 1 mL of 25mM p- nitrophenyl phosphatase disodium salt (pNPP). However, no substrate was supplemented in the control soil. The solution was incubated for 4h at 37°C. Afterward, the reaction was halted by adding 1mL 0.5M CaCl2 and 4 mL of 0.1M tris buffer (pH 12). The mixture was incubated for 2h and assayed for absorbance at 400nm by using p- nitrophenol as a standard. The activity of soil phosphatase enzyme was expressed in µg p-PNP/g soil/hour.

Table 1 Quantitative assessment of PGP traits of *Pseudomonas protegens* CP17

ZnO solubilization (µg/ml)	$ZnCO_3$ solubilization (µg/ml)	Siderophore unit (%)	P solubilization (µg/ml)	IAA production (µg/ml)	ACC deaminase (nmol α- KB hour-1mg-1 protein)	EPS production (mg/ml)	NH_3 Production (µmol/ml)
20.74 ± 1.13	36.38 ± 3.96	66.28 ± 1.46	318.83 ± 16.15	67.17 ± 1.77	67.03 ± 1.63	3.14 ± 0.03	0.103 ± 0.01

Mean ± SE is shown in the table. Each value is mean of three replicates

Statistical analysis

All the data of plant growth promotion and pot trial study were statistically analyzed through statistical program analysis of variance (ANOVA), by using the procedure of general linear model (SPSS, ver. 16.0) to disclose significance in different treatments. Duncan's Multiple Range Test (DMRT) was applied to determine the difference between individual events at significant level of $P \leq 0.05$.

Results

Prevalence of zinc solubilizing bacteria

A total of 10 bacteria were isolated from rice rhizospheric soil. Out of these, only 6 bacterial isolates were capable to solubilize Zn in both forms (ZnO and ZnCO3) with varying solubilization efficiency from 66 to 200%. Two strains viz. CP17 and CP21 showed good solubilizing efficiency on both Zn supplemented medium by producing halo region around bacterial colony thereby designated as potent Zn solubilizers (Figure S1). Since bacterial isolates were isolated from salt affected site, therefore, NaCl tolerance capacity of these isolates were assessed by growing in Nutrient broth ammended with different NaCl concentrations. The optimum tolerance level was determined for CP17 and CP21 at different NaCl concentrations, of which isolate CP17 exhibited maximum tolerance up to 20% of NaCl concentration as shown in the supplementary material (Figure S2). On the contrary, isolate CP21 was unable to tolerate 10% of NaCl concentration. However, 22% (w/v) NaCl concentration showed an inhibitory effect on the growth of CP17. Hence, bacterium CP17 was selected for further studies.

Molecular characterization and functional PGP traits of isolate CP17

Phylogenetic analysis corroborated that isolate CP17 (gene accession number MK942704) was characterized as *Pseudomonas protegens* showing 99.45% similarity with *Pseudomonas* sp. *strain* CV10.2 (Figure S3). Plant growth promoting traits, such as IAA production, phosphate solubilization, ACC deaminase activity, siderophore, EPS, ammonia and HCN production were analyzed to assess the putative plant growth promoting activities of the isolate. Results indicated that isolate was able to produce IAA, siderophore, EPS and ammonia. Activities of ACC deaminase enzyme and phosphate solubilization were also found positive. However, isolate showed negative results for HCN production. The positive values achieved from these assay are mentioned in Table1.

Estimation of zinc solubilization

The bacterial isolate was assessed to measure the magnitude of Zn solubilization on Minimal medium supplemented with different concentrations of NaCl. The impact of bacterial isolate CP17 was assessed on 0.1% supplemented ZnO and $ZnCO_3$ to evaluate the degree of solubilized Zn in Minimal medium. On assessment under in vitro conditions, inoculation of *P. protegens* CP17 solubilized significant Zn content in liquid broth in contrary to uninoculated control. The values attained from AAS for Zn solubilization confirmed a gradual decrease in solubilization efficiency as the concentration of NaCl increased (Fig.1). Among the Zn compounds, solubilization of Zn was found higher in $ZnCO_3$ supplemented medium in contrary to ZnO inoculated with *P. protegens* CP17. The maximum value for $ZnCO_3$ solubilization in *P. protegens* CP17 recorded was 36.38 ± 3.96, 34.37 ± 4.53, 31.57 ± 1.70 and 27.96 ± 1.13µg/mL in 0, 100, 200 and 300mM NaCl (w/v) concentration, respectively. The results signify that the magnitude of Zn solubilization was strongly affected by NaCl concentration in which the bacteria were studied. On the other hand, inoculation of *P. protegens* increased soluble Zn in NaCl supplemented medium which can be attributed to bacterial organic acids production under saline conditions.

Influence of *P. protegens* CP17 in organic acid production and changes in pH pattern for zinc solubilization

On evaluation under in vitro conditions, the CP17 mediated Zn solubilization was attributed by pH reduction and organic acid accumulation. Inoculation with CP17 strains significantly declines the pH pattern of culture medium

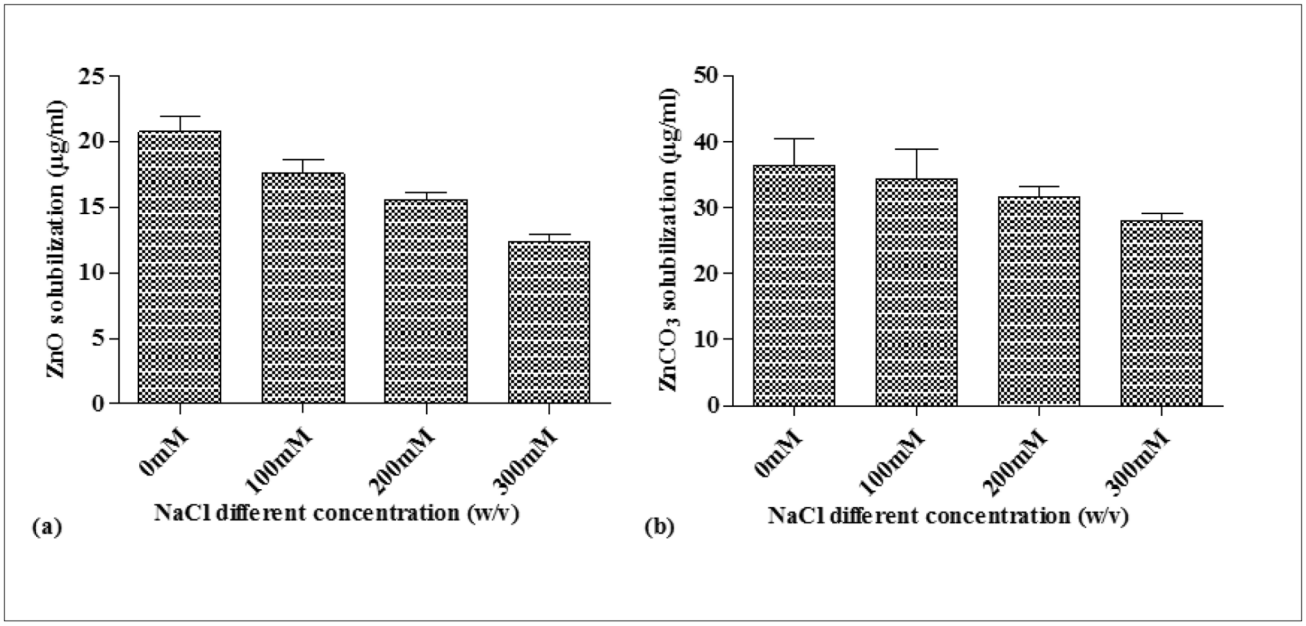

Fig. 1 Quantitative estimation of bacterial zinc solubilization under different NaCl concentrations

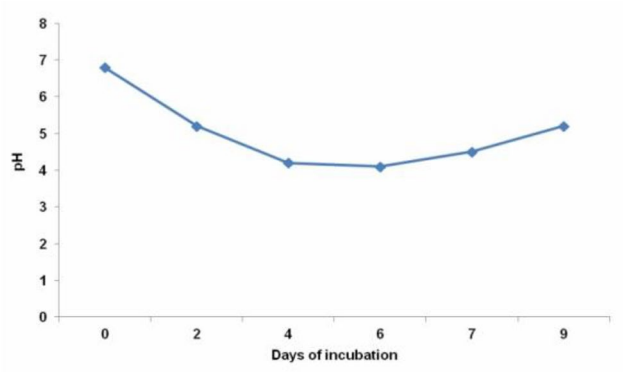

Fig. 2 Changes in pH pattern of minimal media supplemented with 0.1% ZnO implicated with isolate *Pseudomonas protegens* CP17

supplemented with ZnO compound over uninoculated control (Fig.2). Reduction of pH in the medium was also periodically observed up to 9 days. After the inoculation of CP17 bacterium, the initial broth pH (6.8) was reduced up to pH 4.1. Moreover, reduction in the pH pattern of bacterial implicated broth medium was also related positively with organic acids production. The observed acidification in the medium is probably due to the higher accumulation of organic acids produced by *P. protegens* CP17. In total, 15 organic acids were produced in CP17 inoculated medium, which was chiefly involved in solubilization of Zn content. Remarkably, organic acids i.e. malonic acid (180.042), thioacetic acid (296.181), propionic acid (288.111), carboxylic acid (172.074), glutamic acid (413.314) were determined through UPLC coupled with QTOF Mass spectrometry (Fig.3). These results indicate that the bacterial mediated organic acid production was a major mechanism for Zn solubilization and responsible for pH reduction in the medium.

acdS gene amplification in *P. protegens* CP17

The current study also inspected the presence of *acdS* gene in *P. protegens* CP17 through amplification with degenerative primers designed by Blaha et al. (2006). These primers were based on *Pseudomonas* and *Enterobacter* sequences. The alignment of amino acid sequences for selected ACC deaminase and ACCD homologs from a variety of bacterial genera showed that these primers were distinctive to ACC deaminase and predicted that 516 and 750bp amplicons are specific to *acdS* gene, a functional gene which codes for ACC deaminase enzyme. The presence of *acdS* gene in bacterial genomic DNA confirms the activity of ACC deaminase enzyme, which reduced the level of highly produced ethylene in saline conditions and maintained the equilibrium between the host plant and rhizospheric soil by utilizing ammonia and α ketobutyrate as a C and N source.

Effect of *P. protegens* CP17 strain on germination of wheat seeds

Zn plays a crucial role in seed germination and improved seedling ability to grow and endure any ecological conditions. To determine the effects of salinity, various concentrations i.e. 0, 100 and 150 mM of NaCl concentrations were used for seed germination experiment. Results revealed

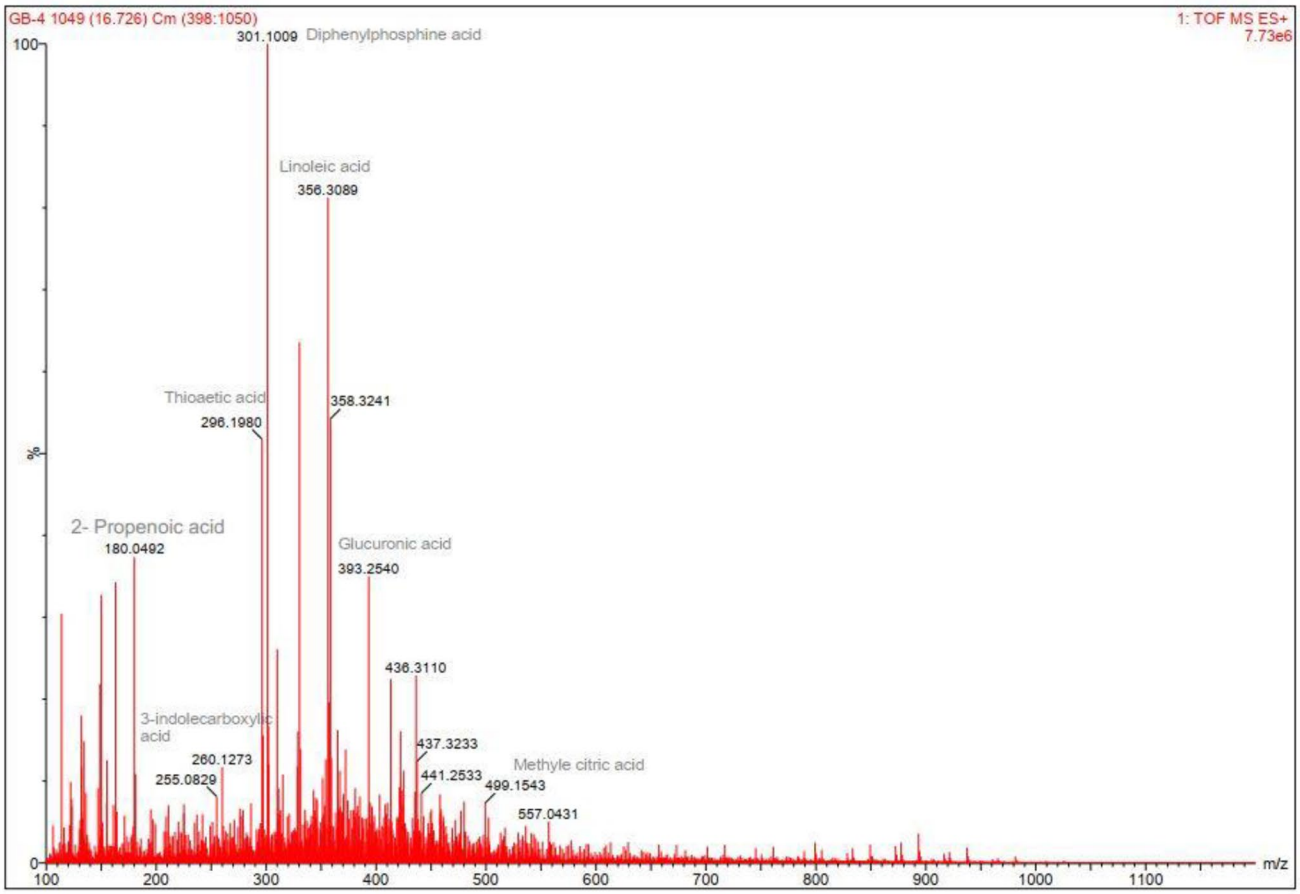

Fig. 3 UPLC-MS chromatogram for organic acids profiling produced by *Pseudomonas protegens* CP17 in zinc minimal broth medium

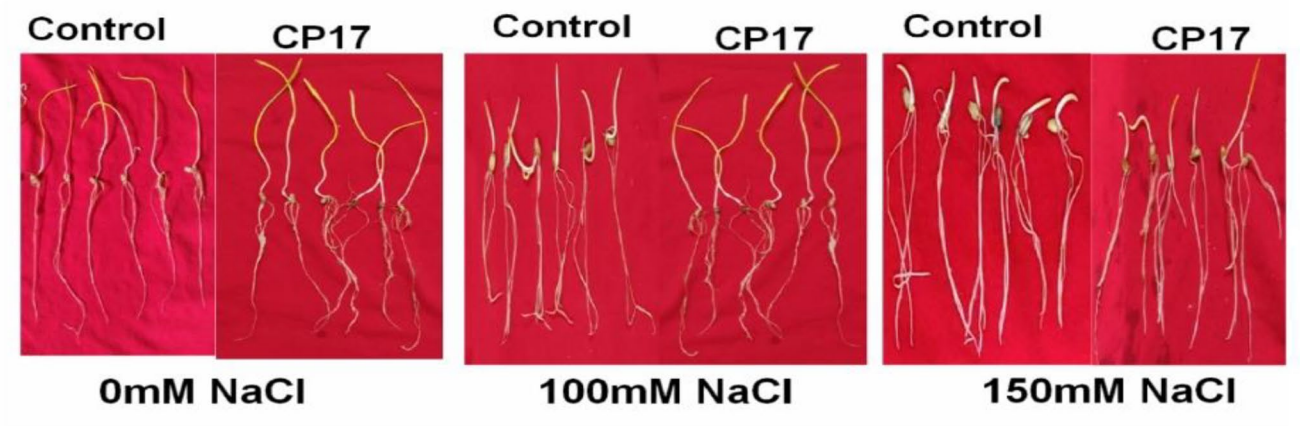

Fig. 4 Comparative effect of *Pseudomonas protegens* CP17 implications on wheat germination efficiency under different NaCl concentrations

that after 8 days of incubation the root length, shoot length and total fresh biomass of seedlings were reduced in different NaCl concentrations (Fig.4). The standard germination of control seeds was reduced gradually from 92.66 ± 1.15 to 91.33 ± 4.16 and 77.33 ± 2.30% in 0, 100 and 150 mM NaCl, respectively. However, the implications of *P. protegens* notably enhanced the germination percent in contrary to control with the value of 97.33 ± 1.15, 97.33 ± 1.15 and 94.66 ± 3.05%, respectively. Similarly, length of shoot and root along with fresh and dry biomass was improved in P. protegens inoculated plants under different NaCl concentrations. These results signify that diminution caused by NaCl was perked up with the implication of *P. protegens* (Fig.4; Table2).

Table 2 Effect of *Pseudomonas protegens* CP17 on germination parameters of wheat seedlings under different NaCl concentration

Treatments	First count (%)*	Standard count (%)*	Shoot length (cm) *	Root length (cm)*	Fresh weight (mg) *	Dry Weight (mg) *
Control (0mM NaCl)	75.33 ± 1.15d	92.66 ± 1.15bc	8.64 ± 0.112d	13.81 ± 0.11e	1832 ± 11.93d	218 ± 9.71c
P. protegens (0 mM NaCl)	87.33 ± 1.15e	97.33 ± 2.30c	13.35 ± 0.152e	17.55 ± 0.08f	2006 ± 9.60f	304 ± 9.07e
Control (100 mM NaCl)	65.33 ± 2.30c	91.33 ± 4.16b	3.96 ± 0.21b	7.85 ± 0.94b	1410 ± 27.39b	139 ± 16.77b
P. protegens (100 mM NaCl)	75.33 ± 1.15d	97.33 ± 1.15c	7.85 ± 0.12c	11.50 ± 0.13d	1908 ± 43.10e	262 ± 32.19d
Control (150 mM NaCl)	35.33 ± 2.30a	77.33 ± 2.30a	2.77 ± 0.08a	5.60 ± 0.172a	1140 ± 28.29a	104.3 ± 1.06a
P. protegens (150 mM NaCl)	47.33 ± 2.30b	94.66 ± 3.05bc	7.61 ± 0.19c	10.93 ± 0.127c	1496 ± 58.87c	156.33 ± 2.23b

Data were analyzed at $P < 0.05$ level of significance. Mean ± SE is shown in the table. Each value is mean of three replicates

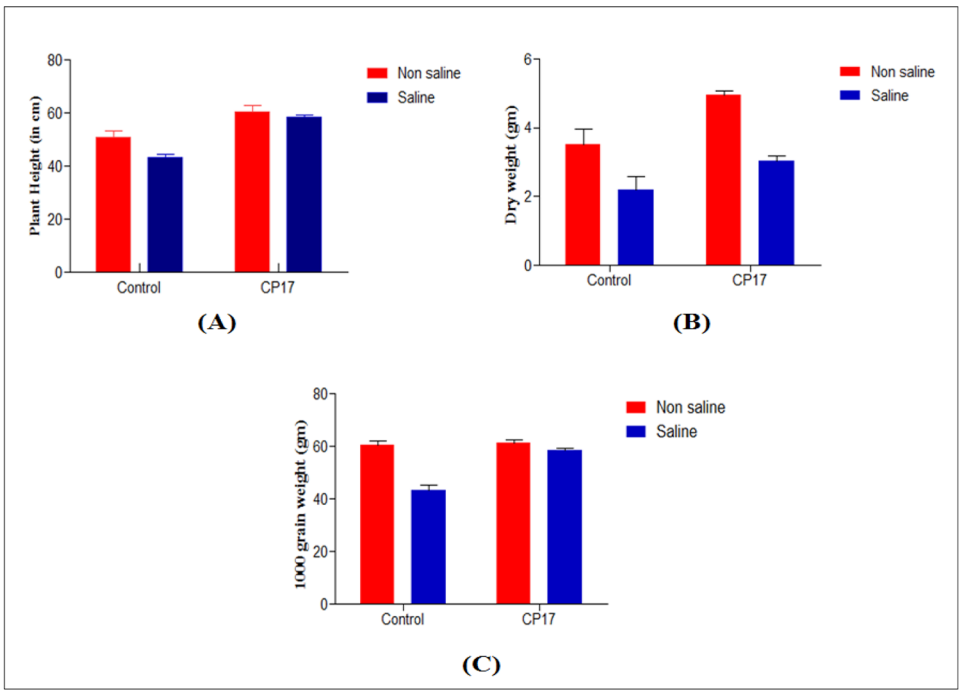

Fig. 5 An overview of different growth parameters in control and *Pseudomonas protegens* CP17 implicated wheat plants in saline and non saline soil. (A) plant height (B) dry weight and (C) 1000 grain weigh

Effect of *P. protegens* CP17 on growth, development, and yield of wheat

The mature wheat plants were harvested and studied for dry biomass, 1000 grain weight, and plant height. A significant increment of 19.10% and 35.01% was observed in the height of wheat plant after the implication of CP17 contrary to control treatment under non saline and saline conditions, respectively. However, in dry weight biomass, an increment of 41.71% and 38.35% were monitored in bacterial implicated plants under non saline and saline soil in contrary to control plants, respectively (Fig.5). Besides this, a marked effect of *P. protegens* inoculation on plant's yield was also observed in both saline and non saline soil. *P. protegens* inoculated plants recorded higher grain, straw yield compared to control plants under both non saline and saline soil as depicted in supplementary material Figure S4. The reduced growth and yield in control plants could be assumed by nutrient imbalance and restriction in water availability due to salinity exposure.

Photosynthetic pigments

Results obtained with the estimation of chlorophyll and carotenoid content in leaf tissues of bacterial treated and non treated wheat plants under saline and non saline stress are summarized in Fig.6. A relative increase in photosynthetic pigments (chlorophyll and carotenoid) for *P. protegens* CP17

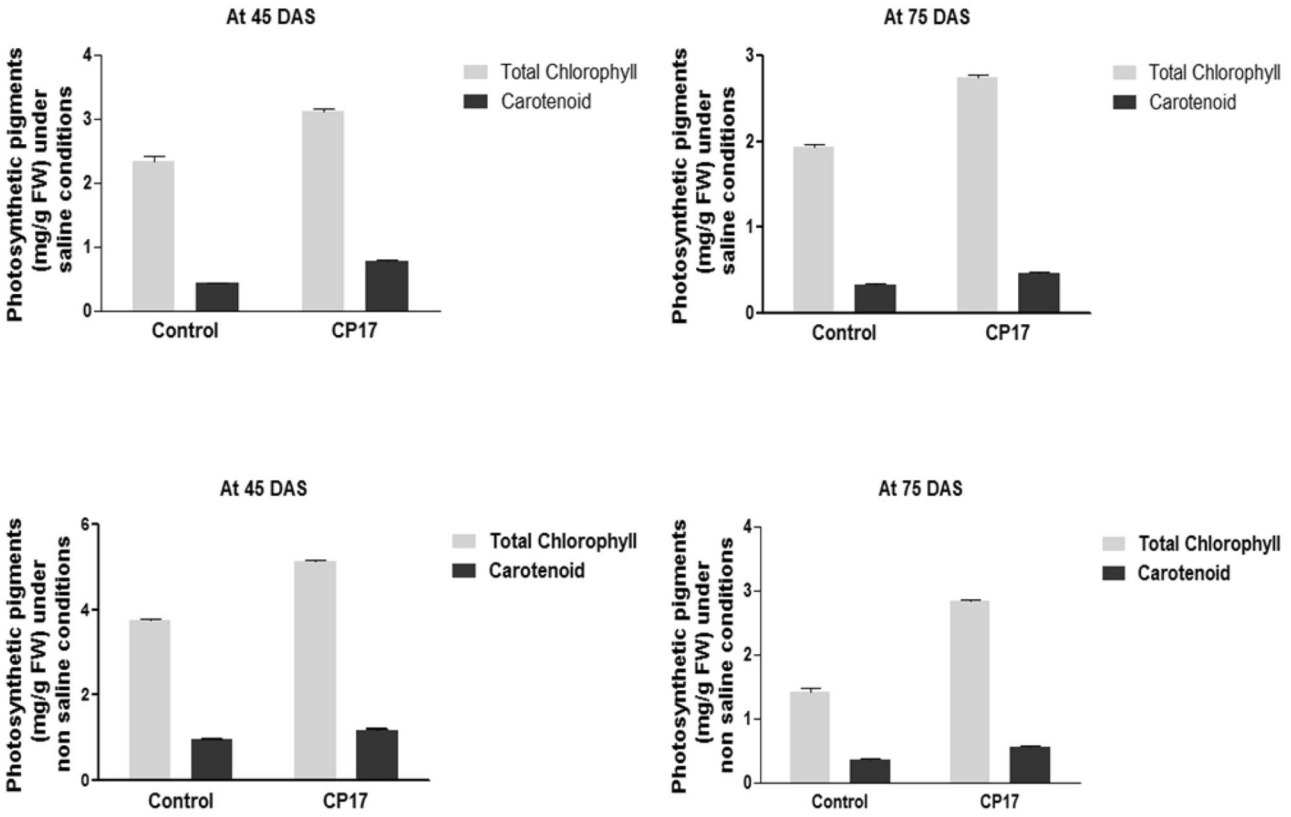

Fig. 6 An overview of wheat photosynthetic pigments under saline and non saline conditions after treatment of *Pseudomonas protegens* CP17

implicated wheat plants was observed. Increased content of chlorophyll (33.19 and 37.33%) and carotenoid (78.76 and 22.80%) was recorded in contrary to control plants under both saline and non saline soil at 45 DAS, respectively. Similarly, at 75 DAS an increment of 41.23 and 99.30% chlorophyll and 38.57 and 54.64% carotenoid content over control was recorded under saline and non saline conditions, respectively. These increased photosynthetic pigments in bacterial inoculated plants could be ascribed to the protective role of bacterium from chlorophyll degradation and this might, in turn, enhance the overall growth and photosynthetic ability of wheat plant. The least photosynthetic ability in uninoculated treatment could be implicit by suppression of chlorophyll synthesis and mineral absorption under saline stress.

Effect on physiological parameters of wheat under salinity and normal soil

A significant change in the proline, MDA (malondialdehyde), catalase, RWC (relative water content, electrolytic leakage) contents were recorded among control as well as *P. protegens* inoculated plants under saline and non saline soil at both 45 and 75 DAS, respectively. A major increment was registered in proline, catalase and relative water content in *P. protegens* implicated wheat plants during exposure of saline stress in contrary to control excluding the lipid peroxidation and electrolytic leakage, which was recorded less in *P. protegens* implicated plants (Fig.7). The present study clearly showed that the application of *P. protegens* markedly increased the various enzymatic activities in contrast to control while alleviating salinity. These osmolytes and antioxidant activities of bacterium played an important role in salinity tolerance and better growth in wheat seedlings under salt stress which were reflected in morphological parameters of wheat seedling growth.

Assessment of zinc uptake in the rhizosphere, shoot, and grains of wheat

Application of *P. protegens* CP17 clearly showed the increased uptake of Zn in plant parts and contrary to uninoculated control, which was reflected through data of enhanced Zn concentration in soil, straw, and grains of wheat plant inoculated with *P. protegens* under saline and

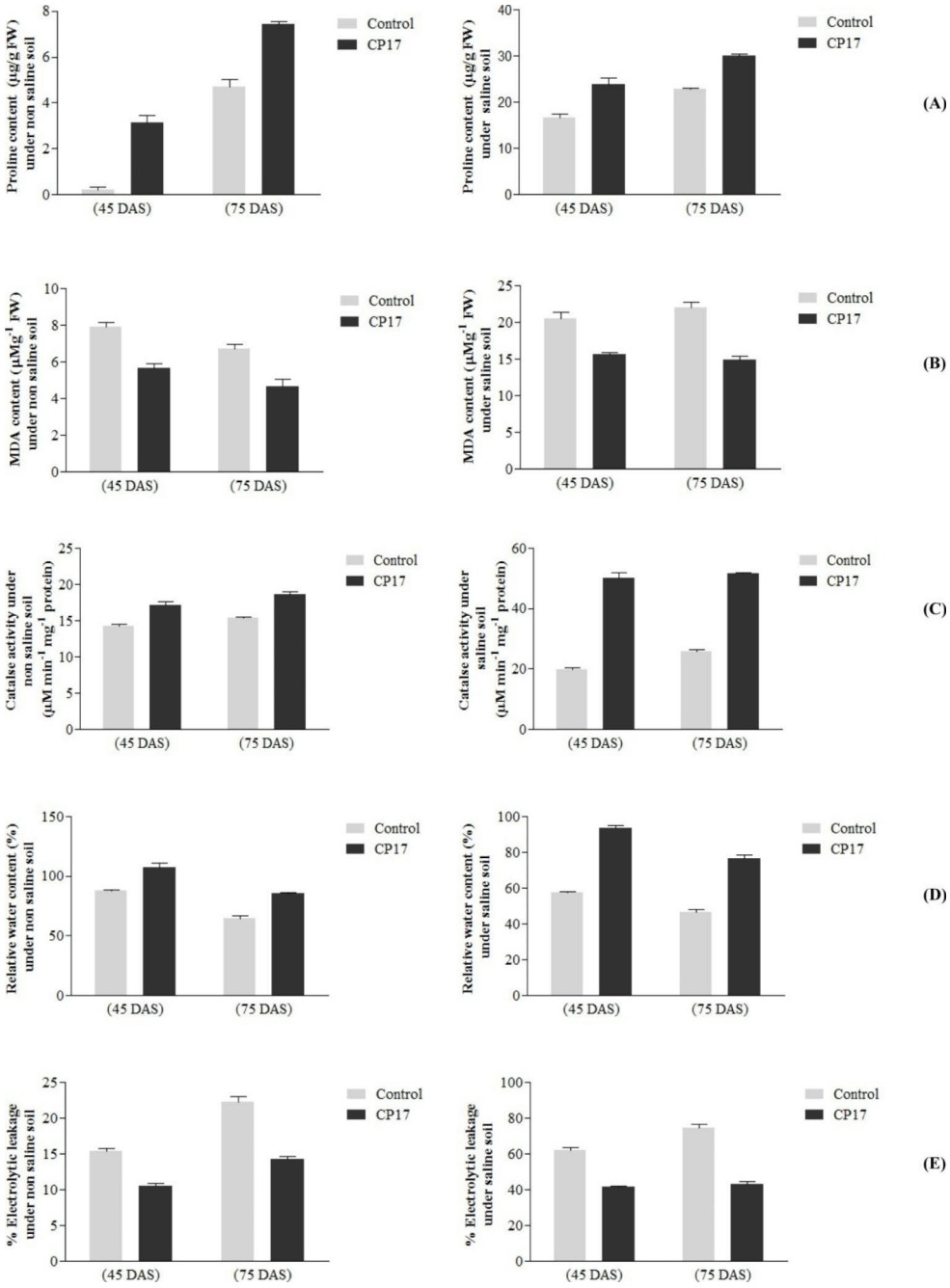

Fig. 7 Effect of *Pseudomonas protegens* CP17 treatment on physiological characteristics of wheat in saline and non saline soil (A) proline content (B) MDA content (C) catalase content (D) relative water content (E) electrolytic content

Table 3 Effect of PSB *Pseudomonas protegens* CP17 and control treatment on zinc content (mg /kg)

S. No.	Available zinc in non saline soil and plant (mg/kg)			Available zinc in saline soil and plant (mg/kg)		
	Soil	Straw	Grain	Soil	Straw	Grain
Control	0.836 ± 0.008	34.36 ± 0.41	22.83 ± 1.25	0.754 ± 0.006	28.80 ± 0.36	16.33 ± 1.04
CP17	1.32 ± 0.008	50.23 ± 0.15	29.33 ± 1.25	0.978 ± 0.0004	47.53 ± 0.15	24.33 ± 0.76

Data were analyzed at $P < 0.05$ level of significance. Mean ± SE is shown in the table. Each value is mean of three replicates

nonsaline soils (Table3). It was observed that *P. protegens* enhanced Zn by 29.70%, 69.03% and 48.98% over control in rhizospheric soil, straw and grains of wheat plant, respectively, under saline stress (Table3). However, in non saline soil, Zn content was increased by 57.89, 46.18, 28.47%, respectively in *P. protegens* inoculated plants over control. The results reflected that application of bacteria mitigates saline toxicity through various enzymatic activities and thus increased the Zn uptake by plants. The bioavailability of the solubilized Zn by *P. protegens* might be due to the

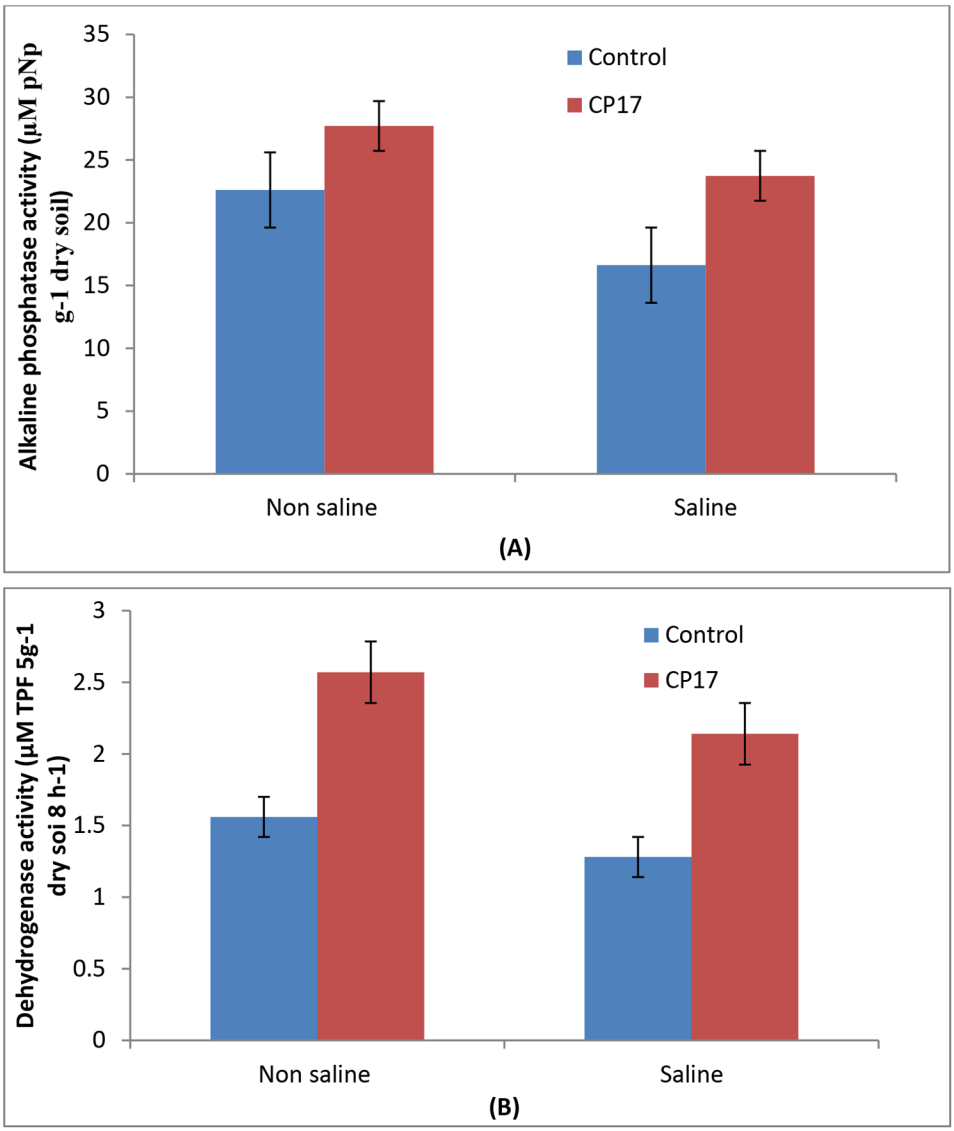

Fig. 8 Effect of *Pseudomonas protegens* CP17 on soil enzymatic activity in wheat rhizosphere under saline and non saline conditions (A) Alkaline phosphatase activity (B) Dehydrogenase activity

production of organic acids by this strain and leading easy Zn uptake by plants for better growth. Our study presumes the significant role of *P. protegens* CP17 inoculation as it increased Zn concentration in different parts of wheat plant under non saline and saline conditions, which is needed for daily human requirements and has large implications in terms of conquering Zn malnutrition in developing world.

Soil enzyme activity

An extended parameter was also assessed to observe the effect of salinity on soil health and microbial functioning by determining soil enzymatic activities. Data represented in Fig.8 indicates that rhizosphere of wheat cultivated in saline soil without the implication of *P. protegens* have lower microbial activity in contrary to control. This result indicates the lethal effect of salinization on soil health which tends to alter the diversity and activities of microorganisms in the rhizospheric region. However, the dehydrogenase activity was subsequently higher i.e., 2.14 and 2.57 µM TPF $5g^{-1}$ dry soil $8h^{-1}$in *P. protegens* inoculated wheat plants under both saline and non saline conditions, respectively. Similar results were also found for soil alkaline phosphatase activity in both bacterial implicated and control plants under non saline and saline conditions. Elevated values of soil phosphatase activity i.e. 23.74 and 27.71 µM pNp g^{-1} dry soil $2h^{-1}$ was deliberated in *P. protegens* treated wheat plant under saline and non saline soil conditions, respectively. This might be due to alterations in rhizospheric enzymatic activities related to Zn cycling, occurred as a consequence of *P. protegens* CP17 inoculation in wheat crops.

Discussion

Rhizobacteria are well recognized for their potential role in plant growth promotion and mitigating different environmental stresses including salinity. Salinization has an undeviating consequence on the chemical, biological and physiological properties of soil which has a detrimental impact on plants productivity. The unpleasant effect of soil salinity on plant growth is because of ion toxicity, osmotic stress, nutrient imbalances or a mixture of all mentioned factors (Bharti et al. 2016). Micronutrient deficiencies are very frequent in salinity conditions because a range of crops is not able to chelate, assimilate or solubilize various micronutrients in high pH and become a foremost reason of micronutrient malnutrition or hidden hunger in such environment (Hu and Schmidhalter 2005). Among micronutrients, Zn is highly essential nutrient required throughout the lifecycle of plants, animals and human being. Zn deficiency has become a severe problem caused by low Zn content of crops, grown in Zn deficient soils affecting 50% of the global population (Hefferon 2019). Keeping this in view, the present study is an effort to evaluate the multifarious role of bacterial isolates for decoding its potential as a Zn biofortifying agent along with improvement of soil fertility, health and nutritional quality of wheat under non saline and saline stress conditions.

In our study, salt tolerant rhizobacteria were isolated from the rhizospheric region of *Oryza sativa*. The isolates were tolerant to 15 to 20% NaCl in Nutrient broth medium and had different potential of insoluble Zn (ZnO and $ZnCO_3$) solubilization. Among all the isolates, bacterium CP17 was recognized as an efficient Zn solubilizer with highest NaCl tolerance capacity. In addition, bacterium CP17 was gram negative, *acdS* positive and characterized as *Pseudomonas protegens*. Various studies have reported that the PGPR isolates belonging to *Pseudomonas* genera are involved in conferring salinity tolerance in different crop species. *P. protegens* has been extensively exploited for its biocontrol efficiency against various phyto pathogens and is associated with plant rhizomes under extreme conditions (Andreolli et al. 2019). However, the biofortifying ability of *P. protegens* has not been evaluated under extreme conditions till now.

In this study, *P. protegens* CP17 was capable to solubilize maximum Zn from different forms of respective ores (ZnO and $ZnCO_3$) in Minimal broth medium supplemented with different concentrations of NaCl. The mechanisms of Zn acquisition by CP17 from insoluble Zn compounds were due to the production of organic acids and pH reduction, and thereby influencing the bioavailability of Zn in the plant. Such solubilization of Zn compounds mediated through organic acids production, pH reduction and subsequent release of available Zn in the external environment had been reported (Costerousse et al. 2017; Upadhayay et al. 2018; Khan et al. 2019).

However, the enhanced NaCl tolerance efficacy of *P. protegens* CP17 could be attributed to its EPS and ACC deaminase production potential. Mitigation of salinity stress by PGPR having ACC deaminase activity has been known to protect the plant against various abiotic stresses by reducing ethylene levels (Penrose and Glick 2003). During stress conditions, the extra amount of ethylene is produced in plant resulting in poor nutrient acquisition and plant growth. The rhizobacteria were able to decrease the level of plant ethylene by producing ACC deaminase enzyme. A significant portion of the ACC exudates from roots is hydrolyzed by bacterial ACC deaminase into α ketobutyrate and ammonia between internal side of the root and rhizosphere, which is utilized as unique source of carbon and nitrogen (Mitra et al. 2018). Similarly, attachments of EPS to Na^+ ions lessen the Na^+ toxicity and making it unapproachable for crops. It has been documented that implications of *Bacillus* and *Pseudomonas* species alleviate the saline stress through EPS production and consequently resulted in enhanced nutrient accessibility and development of various crops (Costa et al. 2018). In this study, *P. protegens* used its ACC deaminase to achieve ACC utilization responsible for indirect diminution of stress by reducing ethylene concentration. Moreover, EPS secretion for structural adjustment during desiccation could be a crucial protection factor, trapping a reservoir of nutrients and water for bacterial survival and hence inducing plant growth and nutrient accessibility. These results verified that application of salt tolerant rhizobacteria promoted better growth of plants under nonstress and saline stress conditions. This suggests that interaction of rhizobacteria ameliorated salinity stress.

Under saline stress, the root and shoot length of uninoculated wheat seedling was significantly declined while inoculation with bacterial strain CP17 enhanced their length. The growth-promoting potential and enhanced NaCl tolerance efficacy of *P. protegens* CP17 could be ascribed because of its IAA production potential (Gontia-Mishra et al. 2017). The longer seedling growth of CP17 inoculated plants under stress and non stress conditions were due to the production of IAA by bacterial isolate. It is well recognized fact that indole 3- acetic acid production is indulged in cellular division and enhancement of lateral as well as adventitious roots which in turn assists the host plant in absorption and assimilation of various micronutrients (Abbas et al. 2019).

Germination, emergence, and early seedlings growth are three stages for crop establishment, which are sensitive to salinity. In current study, *P. protegens* CP17 implicated wheat seedlings showed a noteworthy impact on the standard count and germination speed under saline stress in contrast to uninoculated control. It has been shown that increased

NaCl concentration reduced the standard count germination, length and biomass of wheat seedlings. A recent study done by Nasrin and Mannan (2019) observed that excessive uptake of the salt ions which causes toxicity for the plant and reduced water availability between seeds and outer environment, inhibits the seedling emergence and thus reducing the speed of germination and germination count. Similarly, in situ pot trial experiment registered a prominent increase in dry biomass, plant height, and 1000 grain weight was recorded in bacterial implicated wheat plants in contrary to control. Our results are in correspondence with the literature on salinity tolerance in various crops including wheat through the implication of rhizobacteria (Chang et al. 2014; Bharti et al. 2016). The saline stress stimulates low osmotic potential and thus affects water availability to the plants. The % RWC in control plants was significantly lesser as compare to *P. protegens* implicated plants. This might be because uninoculated wheat seedlings were facing an osmotic challenge for the transpiration process or uptake of water from the soil due to salinity. Chlorophyll level in stressed tissues serve as index for salinity tolerance of tissue. High salinity exposure to plant decreased the chlorophyll, which was noticed in our study at 45 DAS and 75 DAS in control wheat seedlings. Furthermore, the application of *P. protegens* significantly enhanced the photosynthetic content in wheat seedlings. Salinity stress enhances ROS production, leading to severe ROS-associated damage to the chloroplast. If the salt concentration of the saturation extract contains more than 12g/l, the soil is said to be highly saline. However, if the salt concentration of the saturation extracts contains 3–6g/l and 6–12g/l is considered slightly saline and medium saline, respectively. Analogous results were reported by Sapre et al. (2018) mentioned that higher salinity often results in deterioration of chloroplast, which could be the prime reason for chlorophyll degradation under saline stress. However, they have also reported the chlorophyll increment in the bacterial implicated wheat plants under saline stress. The total proline and catalase content were also measured in the present study. Proline is a critical biochemical marker of salinity tolerance and play key function in osmoregulation. In order to combat the oxidative stress developed due to saline toxicity, the activities of antioxidant enzymes increased in *P. protegens* inoculated plants. The present work reported that PGPR inoculated wheat seedlings registered higher proline content in contrast to un-inoculated seedling under saline stress. However, low accumulation of proline level was recorded in the wheat plant under non-stressed soil. It could be implicit that *P. protegens* inoculated wheat seedling did not demonstrated the lethal effect of saline stress due to the accumulation of proline content in plant tissues (Torre-Gonzalez et al. 2017). In the present investigation, a considerable high activity of catalase enzyme was measured in strain *P. protegens* inoculated wheat seedlings compared to control seedlings under saline stress. It could be presumed that PGPR inoculated seedlings did not register much stress due to the high activity of catalase enzymes which scavenge ROS and catalyzes the H_2O_2 decomposition into O_2 and H_2O, without free radicals production. These findings are in agreement with findings of Bharti et al. (2016), where PGPR inoculation decreased the detrimental effects of oxidative stresses in wheat.

An extended parameter of oxidative stress is the Malondialdehyde (MDA) content. It is produced by unsaturated fatty acids oxidation through ROS, which ultimately affects the integrity and functionality of membrane and results in enhanced electrolytes leakage from the cells (Sapre et al. 2018). *P. protegens* inoculated wheat seedlings under saline stress demonstrated remarkably low electrolytic leakage and MDA content from leaf tissues, suggesting that PGPR inoculated wheat seedlings protects the integrity of cell membrane from oxidative injury caused by saline stress (Barnawal et al. 2014). Similar outcomes was also found by Zhou et al. (2009) in which they confirmed that PGPR primed plant had notably less electrolytes leakage from leaf tissues and subsequently maintained cell membrane integrity from the lethal impact of salinity.

At maturity, inoculation of *P. protegens* significantly increased Zn uptake in rhizospheric soil and its assimilation in straw and grains as compare to un-inoculated control in wheat under both the saline and non-saline soils. This functionality of Zn assimilation might be owed to plant-root colonizing ability of PGPR, which helps the plants to acclimatize, adjust and survive under the excessive flux of environmental conditions through diverse mechanisms such as the production of ACC deaminase enzyme, EPS, indole acetic acid and various enzymatic activities (Abbas et al. 2019). These factors are responsible for mitigation of salinity stress and thus improving crop plant nutrient acquisition and health under saline stress conditions. In addition, increased root surface, root length area, root tips, organic acids production and significant drop in rhizospheric pH are major factors accountable for increased Zn accessibility for crop acquirement (Lucas et al. 2014; Shahid et al. 2018). Similarly, soil enzyme activity has been used as indicator for soil health and quality. In this context, effort was made to conclude the rhizospheric enzyme activities are relevant to Zn cycle as a outcome of *P. protegens* inoculation to wheat. The data revealed a significant increment in dehydrogenase and alkaline phosphatase activity with inoculation of *P. protegens* over un-inoculated control in wheat rhizosphere. Elevtaed dehydrogenase activity in rhizospheric soils with *P. protegens* inoculation might increased the availability of biodegradable substrate and improved their microbial activity (Wolinska and Stępniewska 2012). Similarly, higher

alkaline phosphatase activity in saline and non saline rhizospheric soils with *P. protegens* inoculation can contribute to phosphorus nutrition in plants and microbes, which provides simple sugars for the soil microbial population and is explicitly related to soil functions such as nutrient cycling under extreme conditions (Aon et al. 2001).

Keeping in mind the above facts, it is currently well understood that salinity is a combination of various ionic and osmotic stresses which affect plant development, grain quality and soil fertility. The negative impact of salinity not only disturbs nutrient cycling but also affects the micronutrient availability in plant and subsequently, it becomes a prime reason for micronutrient deficiency (malnutrition) in developing countries (Bouain et al. 2014). The increased Zn concentration found in present study has large implications in provisions of conquering Zn malnutrition of developing countries in rapidly changing environments, wherein Zn malnutrition is widespread (Ramesh et al. 2014; Hefferon 2019). Similarly, an increased Zn concentration in wheat grains with bacterial inoculation also signifies the relevance of bacterium *P. protegens*, which could be a superlative applicant for the development of multifarious characteristics for Zn biofortification by ameliorating the plant health under abiotic stress conditions.

Conclusion

Our study demonstrated that inoculation of bacterial strain *P. protegens* CP17 takes part in biofortification of Zn by improving the biochemical and physiological status of wheat plant and aided them to tolerate salinity stress. The potent Zn solubilizer *P. protegens* showed salt tolerance up to 20% NaCl and able to produce exopolysaccharide and ACC deaminase enzyme along with other plant growth promoting traits, suggesting their role in attenuating the effect of salinity stress. Therefore, the selected bacterial isolate improved soil fertility, plant growth, nutrient accessibility and protected wheat plants from the negative impact of salinity. In addition, the bacterium *P. protegens* CP17 was able to solubilize Zn up to the level of 300mM NaCl concentration by reducing pH and producing various organic acids under in vitro conditions and ameliorating Zn content in rhizospheric soil, straw and grains of wheat in saline and non saline soils under in situ conditions and proving itself a potential biofortifying agent. Therefore, plant inoculation with *P. protegens* CP17 could be a sustainable option to develop salinity tolerant crop to improve not only germination, growth and soil fertility but also to improve the nutrient quality of grains and to alleviate salinity stress persuaded injury in wheat plants. Moreover, as per our knowledge, this is the first investigation providing a detailed mechanistic view of bacterial mediated Zn biofortification in wheat under saline conditions. However, further experiments need to be undertaken to assess the efficiency of this strain under core field conditions to overcome Zn malnutrition in developing countries.

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Declarations

Statement and Declaration No grant has been received for the current research work. The author declares no conflict of interest.

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