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RESEARCH ARTICLE

# Priming with ACC-utilizing bacterium attenuated copper toxicity, improved oxidative stress tolerance, and increased phytoextraction capacity in wheat

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## Abstract

The major challenges for the plants growing in metal-contaminated soils are deficiency of nutrients, biomass reduction, and severe oxidative damages in the presence of heavy metals. In this regard, our aim was to overcome these challenges through the use of efficient microbial strains in metal-polluted soils and to assess its/their physiological and biochemical effects. In the current study, a copper (Cu)-resistant bacterium was isolated from the rhizospheric soil of '*Ziziphus nummularia*' and evaluated for its ability to promote the wheat growth under the gradient stress of copper. Based on 16S rRNA gene sequencing, the isolate was identified as *Pantoea* sp. Among the plant growth promoting tests, the isolate showed the production of indole acetic acid, solubilization of inorganic phosphate, and ACC deaminase activity. Also, the isolate showed resistance to many heavy metals and antibiotics and increased the water-soluble copper in solution. The results of pot studies showed that bacterial application promoted various growth parameters of wheat plants and also enhanced the Cu uptake of wheat from the Cu-amended soil. The results showed that enhancement of Cu stress (100 to 300 mg $kg^{-1}$) resulted in a decrease in various compatible solutes such as proline, total soluble sugars, and total protein content, and increase in the level of malondialdehyde (MDA), latter of which is the indicator of oxidative stress. Bacterial treatment markedly increased the proline, soluble sugar, total protein content, and decreased the MDA content under Cu stress. In addition, bacterial inoculation significantly alleviated the harmful effect of metal toxicity by decreasing the activation of ROS molecules including superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$). The activation of various antioxidative enzymes such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) was noted following bacterial inoculation under Cu stress. Therefore, the present study demonstrates the potential of the isolate *Pantoea* sp. ZNP-5 to improve the growth and phytoextraction of metal from the metal-polluted soil through the polyphasic mechanism of action.

**Keywords** ACC deaminase · Metal stress · Phytoextraction · Osmolytes · Antioxidants

## Introduction

Heavy metal pollution in soils imposes a great threat to the environmental and human health. It can decrease not only the soil microbial activity and plant yield, but also may spread to the surrounding areas (Fellet et al. 2011; Mclaughlin et al. 1999). An elevated level of metal toxicity resulting from the use of agrochemicals, industrial activities, and deposition of sewage sludge pose a threat to the human health by altering the food chain. Among other heavy metals, Cu is a potent pollutant that accumulates in soils and sediments (Lamb et al. 2009). The copper (Cu) is a trace element for all life forms on earth; however, at high concentrations, it becomes toxic (Ouzounidou 1995). Its accumulation in the soil also adversely affects the microbial community, inhibits plant growth, causes leaf chlorosis, and alters the photosynthetic apparatus. Therefore, there is an urgent need to develop the remediation strategy that can effectively remove the toxicity of metals in soil for ecological conservation and safety of human health. Previous reports have shown the importance of metal-accumulating plants that are commonly used in the

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removal of metal toxicity from soils. However, most of the hyper-accumulators are not efficient in accumulating metals at field level due to their slow growth as well as unavailability of metal for root uptake by field-grown plants (Belimov et al. 2005; Rajkumar et al. 2006). Also, low bioavailability of metal in soils also limits the efficiency of phytoremediation (Chen et al. 2005; Sheng and Xia 2006).

The abnormal upsurge of reactive oxygen species (ROS), such as superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals ($OH^-$), is a rapid response to excessive metal stress exposure. The continuous generation of ROS can be extremely harmful as it oxidizes protein, lipid, nucleic acids, and carbohydrates and causes alteration of metabolic pathways (Mittler 2002).Therefore, the increased level of metal proportion in soil impairs the plants metabolic activity, physiological, and developmental response and severely affects the growth. Antioxidative defense mechanisms play a key role to enhance plant oxidative stress tolerance. The important antioxidative enzymes include superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX). SOD plays a critical role in the first line of defense by catalyzing the dismutation of $O_2^-$ into $O_2$. At the same time, the concerted action of CAT and POX decreases the level of toxic $H_2O_2$ by degrading it to water and molecular oxygen. However, with the increase in the severity of stressors, the sufficient antioxidant capacity may not be available to overcome the deleterious effects of oxidative injury with the balance of ROS shifting, which in turn lead to metabolic abnormalities, membrane damage, loss of pigments, and inactivation of enzymes (Roychoudhury et al. 2012).

Under the circumstances mentioned above, the certain rhizospheric microorganism can augment the growth of associated plants and its tolerance to heavy metal, significantly improving the phytoremediation efficiency (Glick 2003). It has been shown in previous studies that inoculation of plants with metal resistant/mobilizing bacteria significantly improves the metal phytoremediation process (Ma et al. 2011; Hrynkiewicz and Baum 2013). Thus, the use of metal-solubilizing rhizospheric bacteria could be used as a promising approach to increase the metal bioavailability in soils as well as to decrease its effect on host plants in heavy metal amended soils. The rhizosphere constitutes the dynamic and complex microenvironment where the microorganisms form the unique communities for detoxification of potentially lethal heavy metals. Microbial population under such environments affects the metal mobility through various mechanisms like acidification, redox behavior and release of chelators (De Souza et al. 1999; Smith and Read 2010).

Plant growths under metal polluted soils are severely affected by the accumulation of metal-induced 'stress ethylene' level. Toxic effect of stress ethylene is manifested by various symptoms like chlorosis, wilting, senescence and growth inhibition. Microorganism with ACC deaminase (1-aminocyclopropane-1-carboxylate) activity, breaks down the precursor of 'stress ethylene' ACC (1-aminocyclopropane-1-carboxylic acid) to α-ketobutyrate (KB) and ammonia and thus help plants to combat with metal stress (Glick et al. 1995; Rajkumar et al. 2006). In addition to ACC deaminase activity, microbes able to solubilize the inorganic phosphate and produce the indole acetic acid that may improve plant competitiveness and its physiological response to external stress factors (Egamberdiyeva and Höflich 2004; Rajkumar et al. 2013a). The presence of these beneficial features prevents stress ethylene-mediated reduction of plant growth and biomass under stressful conditions (Grichko and Glick 2001; Penrose and Glick 2003). Increase in growth of Canola plant '*Brassica napus*' and alleviation of metal stress was observed after inoculation with a metal-resistant ACC utilizing bacterium *Rahnella* sp. JN6 (He et al. 2013). Similarly, *Cicer aritenum* grown in arsenic polluted soil in the presence of metal immobilizing plant growth promoting bacterium *Acinetobacter* sp. nbri 05 showed better growth and higher phytostabilization potential. Plant growth-promoting *Acinetobacter* sp. CC30 significantly enhanced the plant biomass and increased the phytoextraction of Cu by sunflower (*Helianthus annus*).

Use of plant growth-promoting bacteria helps plants to combat the metal stress (Sessitsch et al. 2013). However, little is known about the role of metal solubilizing PGPR on the phytoextraction of Cu from the metal-polluted soils. Since Cu is one of the most widespread contaminants in the environment, it is essential to expand our understanding towards its possible detoxification mechanisms. To the best of our knowledge, few studies have been conducted on the effect of PGPR. Therefore, the present study aimed to characterize metal resistant and mobilizing bacteria to increase the plant biomass, chlorophyll content, and Cu uptake under metal stress conditions as well as to improve the efficiency of phytoextraction of Cu-polluted soils. Moreover, the effect of selected PGPR on the biochemical parameters, antioxidant activities, and oxidative damages was also investigated. The present work can be helpful to evaluate the microbe-mediated detoxification mechanism adopted by the wheat plant in Cu-polluted soils.

## Material and methods

### Isolation of a Cu-tolerant strain

The rhizospheric soil of '*Ziziphus nummularia*' growing in the Shekhawati region of Rajasthan, India (28.13°N, 75.4° East) was collected and brought to the laboratory for study. '*Ziziphus nummularia*' locally known as 'Jharber' is a shrub, which grows primarily in an arid region, and is well adapted to stressed environments. Serially diluted (up to $10^{-9}$) soil sample was spread over sucrose minimal salt low-phosphate (SLP) medium amended with different concentrations of Cu

($CuSO_4$, 100 to 300 mg $l^{-1}$). The plates were incubated for 48 h at 30 °C in an incubator. The appearance of metal-resistant colonies was further streaked and tested for their ACC utilization ability by sub-culturing on solid DF-salt minimal medium amended with 3 mM ACC (Sigma-Aldrich, USA) and incubated for 48 to 72 h at 30 °C. The isolate ZNP-5 was selected based on its Cu tolerance as well as successive growth on the DF-ACC plate. To check its metal mobilizing activity, the isolated organism was tested for its ability to increase the water-soluble Cu, Zn, Ni, and Cd in soils. A mixture of clay and sandy soil (3:1) collected from a non-fertilized farm was supplemented with 200 mg $kg^{-1}$ concentration of each heavy metal and mixed thoroughly. The metal-supplemented soils were allowed to equilibrate for a period of 1 month in the greenhouse. The test isolate was grown in LB medium, and optical density (OD) was adjusted to 1.5. Afterward, the cultures were centrifuged, washed in phosphate buffer (pH 7.0), and re-suspended in sterile water. The bacterial culture (1 ml) was added to the 20 g of metal-contaminated soils in the 250-ml Erlenmeyer flasks and incubated at 30 °C for 1 week. The same amount of boiled culture was added to the 20 g of sterilized soil to be used as a control. For each set of treatments, three replicates were used. The concentration of metal in the filtrate was determined by atomic absorption spectrophotometer.

## Microorganism identification and phylogenetic analysis

The luxuriant growth of the test isolate illustrated its resistance to Cu. For identification of the selected Cu-tolerant ZNP-5, 16S rRNA gene sequence was amplified, sequenced, and analyzed. For this, total genomic DNA was extracted using a genomic DNA extraction kit (Quiagen, USA) and the 16S rRNA gene was amplified using universal primer 27 F1 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1494 Rc (5′-TACGGCTACCTTGTTACGAC-3′). The preparation of the reaction mixture and thermal condition of PCR (polymerase chain reaction) was followed as per previous standardized protocol (Singh et al. 2015). The 1.5 kb size of PCR amplicon was sequenced at Xcelris Genomics Labs Ltd. (Xcelris, Ahmedabad) India, and obtained nucleotides were matched against GenBank database and deposited in the NCBI database. The sequence was aligned using CLUSTAL-X, and a phylogenetic tree was constructed by the neighbor-joining method (bootstrap value 1000) by using Software MEGA6.0.

## Heavy metal resistance of the isolate

Metal resistance ability of isolate was tested by growing in increasing concentration of metal cations added to SLP medium. The various metal ions used in the experiment were Cu as $CuSO_4$, Ni as $NiSO_4$, Cd as $CdSO_4$, and Zn as $ZnSO_4$. Stock solution in the range of 200–1200 g $L^{-1}$ of each metal salts was prepared separately. The selected isolate was streaked on SLP-agar plate supplemented with different concentration of metals. Plates were incubated at 30 °C for 5 days in triplicate sets. The plate without metal ions was used as a control. Further, the growth behavior of test isolate in the metal-contaminated medium was also determined based on their minimum inhibitory concentration (MIC). Culture flasks containing 20-ml LB medium supplemented with heavy metals at the concentration of 250 mg $l^{-1}$ were inoculated with test organism (OD 1.5) and incubated on a rotary shaker at 200 rpm for 40 h at 30 °C. The growth was measured at every 5 h at 600 nm in a UV-Vis spectrophotometer (Jasco Corporation, Japan).

## Test for plant growth-promoting traits

The selected isolate was tested for the presence of plant growth-promoting features like ACC deaminase activity, phosphate solubilization, production of indole acetic acid (IAA), ammonia, and siderophore. For estimating ACC deaminase activity, the selected isolate was cultured in a nutrient medium, and the resulting biomass was suspended in a minimal medium (DF, Dworkin and Foster 1958) with the induction of ACC (3 mM). The ACC deaminase activity was performed as per the protocol described by Singh et al. (2015). The isolate was screened for its solubilization of inorganic phosphate by growing it on NBRIP medium following the method of Mehta and Nautiyal (2001). The ability to produce phytohormone IAA was estimated spectrophotometrically by Salkowsky's reagent assay (Gordon and Weber 1951). The ability to produce ammonia was qualitatively evaluated by Nessler's reagent method (Cappuccino and Sherman 2002). Further, the isolate was tested for arginine decarboxylase production by growing it MDAM (Moeller's-decarboxylase agar medium) supplemented with L-arginine-monohydrochloride (1 g $l^{-1}$) and phenol red (0.02 g $l^{-1}$) used as the pH dye indicator. The ability of the bacterium to produce siderophore was tested by spot inoculating the test organism (1 μl) on chrome azurole S (CAS) agar plates. After growth on CAS-agar plate, it was observed for the presence of yellow or orange halo zone around the bacterial growth.

## Morphological and biochemical characterization

The test isolate was screened for basic biochemical and microbiological tests as per the method described by Harley and Prescott (2002). The carbohydrate utilization ability was tested by carbohydrate utilization test kit (KB 009, Himedia). Test for its resistance to standard antibiotics namely gentamicin (30 μg), ampicillin (10 μg), erythromycin (10 μg), kanamycin (5 μg), tetracycline (10 μg), streptomycin (25 μg), and

chloramphenicol (10 μg) was assayed by the antibiotic disks (HTM 002, Himedia).

### Test of plant growth under Cu stress

The strain ZNP-5 was tested for its ability to promote the plant growth and metal (Cu) uptake in wheat under gradient metal (Cu) stress. The wheat (*Triticum aestivum* L) seeds were properly sterilized with the bleaching solution (NaOCl) and washed with Milli-Q water to remove any traces of bleach solution. For the pot experiment, a mixture of clay sandy soil (3:1) was used. The basic soil properties were: electric conductivity 0.61 ± 0.19 $dSm^{-1}$, pH 6.27 ± 0.08, organic matter 11.30 ± 0.17%, cationic interchange coefficient 7.10 ± 1.08 c mol $kg^{-}$, total phosphorus 10.8 ± 0.35 mg $kg^{-1}$, total sulfur 9.5 ± 0.8 mg $kg^{-1}$, total iron 31.4 ± 4.7 mg $kg^{-1}$, and total copper 1.90 ± 0.06 mg $kg^{-1}$. After sterilization, the soil was mixed with an aqueous solution of $CuSO_4$ to achieve the desired concentration of 100, 200, and 300 mg $kg^{-1}$ and left for 7 days for metal stabilization. Bacterial inoculum was adjusted to $2 \times 10^8$ cells $ml^{-1}$ in phosphate buffer (pH 7.2). Sterilized seeds were soaked in bacterial suspension for 1 h under dark condition. Following bacterization, seeds were sown in plastic pots filled with 400 g of soil in a controlled environment of growth chamber with 16/8 light/dark regimes at 24 ± 2 °C with a humidity of 65–70%. The experiment was conducted for 21 days. Each experiment was performed in triplicate sets.

### Quantification of Cu accumulation and biomass

After 21 days of Cu treatment, plant samples were washed with de-ionized water and were rinsed with 5 mM $CaCl_2$ to remove absorbed metals, if any. Plant tissue was oven dried at 80 °C, grounded, and digested in a mixture of $HNO_3$ and $HClO_4$ (3:1, *v*/*v*). The powdered dry samples (0.5 g) were digested with 4 ml of $HNO_3$/$HClO_4$ (3:1, *v*/*v*), suspended in 10 ml of 0.1 M $HNO_3$, and diluted to 10 ml with distilled water. The Cu contents were determined by using atomic absorption spectrophotometer at the National Horticultural Research and Development Foundation (Nashik, India). Each sample was analyzed in triplicate for accuracy.

### Determination of photosynthetic pigment

For chlorophyll (a and b) estimation, 0.5 g leaf tissue was homogenized in 10 ml of 95% ethanol in the dark and spun at 5000 g for 15 min. The absorbance of the supernatant was measured at 450, 645, and 663 nm in a UV-Visible spectrophotometer (Jasco, Japan), as described by Arnon (1949) and concentrations were expressed as mg $g^{-1}$ fresh weight (Chl a = $12.7A_{663}$–$2.59A_{645}$, Chl b = $22.9A_{645}$–$4.67A_{663}$).

### Antioxidative enzyme assay

A 0.5 g plant leaves were crushed homogenously in 5 ml of 50 mM phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone for SOD and POX and in 0.1 mM EDTA and 12.5 mM $H_2O_2$ for CAT assay. The crude macerate was centrifuged at 10,000*g* for 15 min at 4 °C, and the obtained supernatant was used for the antioxidant assay. For superoxide dismutase assay, the reaction mixture containing 100 μl enzyme extract, 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA was incubated to observe for the development of purple colorformazan, which was then measured at 560 nm against the blank.

The peroxidase (POX) mixture consisted of 100 μl of enzyme extract with 0.1 M phosphate buffer, 0.1 mM pyrogallol, and 5 mM $H_2O_2$. The assay mixture was incubated for 5 min at 25 °C. The absorbance of indigo color formed was read at 420 nm against blank. Catalase mixture (3 ml) consisted of 100 μl enzyme extract with 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA and 12.5 mM $H_2O_2$. Calculation of CAT activity was based on an extinction coefficient of 0.04 $mM^{-1}$ at 240 nm. A unit of enzyme activity was defined as a variation of 0.01 in the absorbance at 240 nm per min.

## Biochemical analysis of plants treated with bacterium under Cu-stress

### Lipid peroxidation

The malondialdehyde (MDA) content represents the degree of membrane damage under metal stress regarding lipid peroxidation. The extent of lipid peroxidation was determined by estimating the malondialdehyde (MDA) content produced by the thiobarbituric acid (TBA) method with minor modification (Hodges et al. 1999). Briefly, leaf tissues (0.5 g) were extracted in 80% ethanol and 1 ml of alcoholic extract was mixed with 1 ml of 0.5% (*w*/*v*) thiobarbituric acid (TBA) containing 20% (*w*/*v*) trichloroacetic acid (TCA). The mixture was incubated at 95°C for 30 min and cooled in an ice-bath. After cooling, the sample was centrifuged at 4500*g* for 15 min, and the absorbance was measured at 400, 532, and 600 nm. The MDA concentration was determined by its molar extinction coefficient (155 $nm^{-1}$ $cm^{-1}$) after subtracting the nonspecific absorbance.

### Proline estimation

For proline analysis, fresh leaves of 0.5 g were homogenized in 3 ml of 5% (*w*/*v*) sulfosalicylic acid and centrifuged at 8500*g* for 10 min. The resulting supernatant of 500 μl was mixed with water and 2% ninhydr in 1:2 ratios. After incubating at 100 °C for 30 min, an equal volume of toluene (2 ml)

was added to the mixture, and upper aqueous phase was used for taking absorbance at 520 nm in a spectrophotometer (Jasco Corporation, Japan). The proline content was measured by comparing the absorbance with a standard curve plotted from the defined concentration of L-proline (Bates et al. 1973).

### Total soluble sugar and protein content

The total soluble sugar (TSS) content was determined using anthrone as per the method of Irigoyen et al. (1992), and the values were calculated using glucose as a standard (20–400 μg $ml^{-1}$) to quantify soluble sugar synthesized in plants. A 0.1 ml of alcoholic leaf extract was mixed with 3 ml freshly prepared anthrone reagent and placed in a boiling water bath for 10 min. The absorbance of the resultant sample was measured at 620 nm in a UV-Vis spectrophotometer (Jasco, Japan).

The total protein content was quantified by the Bradford method (Bradford 1976).Plant tissue of 0.5 g was homogenized in 3 ml of extraction buffer containing 50 mM Tris-HCl (pH 8.3), 1 mM EDTA, 3 mM DTT, 0.08% ascorbic acid, 1 mM PMSF (phenylmethanesulfonyl fluoride) in a pre-chilled mortar. After centrifugation at 10,000*g* at 4 °C for 20 min, the absorbance of the supernatant was measured at 595 nm to evaluate the total protein content present in each treatment.

## Measurement of metal-induced oxidative damage

### Detection of hydrogen peroxide ($H_2O_2$)

The content of $H_2O_2$ in control and bacterium-treated plants was measured under different concentrations of Cu stress (Jana and Choudhuri 1981). Leaf tissue (0.2 g) was macerated homogenously in 50 mM phosphate buffer (pH 6.5), and 3 ml of the solution was then mixed with 1 ml of 0.1% titanium sulfate prepared in 20% $H_2SO_4$. The development of yellow color indicated a positive test for $H_2O_2$, whose intensity was measured at 410 nm. An extinction coefficient of 0.28 $\mu M^{-1}$ $cm^{-1}$ was used to estimate the amount of $H_2O_2$ and expressed as μmol $g^{-1}$ fresh weight.

### Estimation of membrane stability index

For measurement of MSI, leaf tissues were heated in two sets in 10 $cm^3$ of double distilled water (DDW), one at 40 °C for 30 min while the other set at 100 °C for 10 min. The electrical conductivity was measured (C1 and C2) on a conductivity meter, and MSI was calculated using the formula:

$$MSI = [1-(C1/C2)] \times 100$$

### Determination of the formation rate of $O_2^-$

To measure the $O_2^-$ content, leaf tissue (0.5 g) from different treatments was ground in liquid nitrogen. The powdered material was suspended in phosphate-buffered saline (PBS) buffer (50 mM, pH 7.8) and centrifuged at 10,000*g* for 15 min. The obtained supernatant was used for $O_2^-$ content measurements.

### Statistical analysis

The data were analyzed by standard statistical software (SPSS 12.0), and values were presented as the mean ± SD of three replications. The mean difference comparison between the control group and experimental group was analyzed by analysis of variance (ANOVA) and subsequently by Duncan's multiple range test ($p = 0.05$).

## Results

### Identification and PGP features of test isolate

During isolation, 21 native bacterial strains were isolated from the rhizospheric soil of '*Ziziphus nummularia*.' Among these, a potential bacterial strain ZNP-5 was found to tolerate high concentration of Cu. Compared to uninoculated control, ZNP-5 inoculation significantly increased the concentrations of water-soluble Cu, Zn, Ni, and Cd by 10.5-, 4.3-, 2.60-, and 2.10-fold, respectively (Fig. 1). Based on 16S rRNA gene sequence, the isolate was identified as *Pantoea* sp. The obtained sequence of the isolate was submitted to GenBank database under the accession number KJ950706. Phylogenetic analysis revealed that ZNP-5 is closely related to other *Pantoea* sp. and *Pantoea ananatis* (Suppl. Fig. 1). The test strain ZNP-5 showed luxuriant growth on the ACC utilizing plate. The colorimetric assay of the ACC deaminase activity demonstrated production of 135.20 ± 0.010 EU nmol of α-KB $mg^{-1}Prh^{-1}$. Quantitative estimation of phosphate solubilization was carried out in NBRIP medium, which showed phosphate solubilization of 13.35 ± 3.05 μg $ml^{-1}$ after 72-h incubation. The isolate showed IAA production of 0.270 ± 0.036 μg $ml^{-1}$ in the tryptophan-supplemented medium. The appearance of the orange-colored zone on the streaked CAS agar plate illustrated the siderophore production ability of strain ZNP-5. The isolate was also observed positive for ammonia production and argininedecarboxylase test.

### Biochemical analysis of *Pantoea* sp. ZNP-5

Based on the biochemical analysis, it was found as Gram-negative bacteria and positive for catalase, Voges-Proskauer (VP), citrate utilization, lipase, urease, and nitrate reductase.

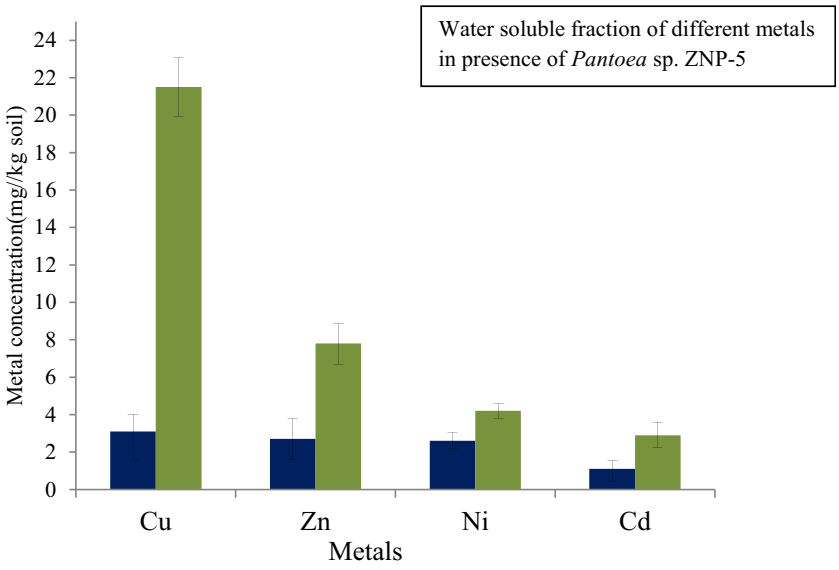


**Fig. 1** The influence of *Pantoea* sp. ZNP-5 on the water soluble fraction of different metals. Error bars are ±standard deviation ($n = 3$)

Among the other tested features, it was found negative for indole, methyl red (MR), and amylase tests. The isolate was found to utilize various carbon sources, and it has been summarized in Suppl. Table 1. Isolate was found to be resistant to ampicillin, vancomycin, streptomycin, kanamycin, gentamicin and sensitive to tetracycline, chloramphenicol (Suppl. Table 2).

## Metal resistance profile of *Pantoea* sp. ZNP-5

The test isolate showed a varied degree of resistance to heavy metals. The strain showed resistance against 700 mg Zn $l^{-1}$, 500 mg Ni $l^{-1}$, 900 mg Cu $l^{-1}$, and 400 mg Cd$l^{-1}$. Among the heavy metals, Cu seems to be less toxic to the strain, followed by Zn and Ni. The order of toxicity of the metals to the strains was found to be Cd > Ni > Zn > Cu. Further, the growth behavior of test strain in the metal supplemented medium was determined to evaluate its capacity to survive in an unfavorable condition. It is evident from Fig. 2 that ZNP-5 exposed to 250 mg $l^{-1}$Cu showed the growth pattern as compared to control. A decrease in the overall growth of ZNP-5 was observed under other metal stress exposure (Zn, Ni, Cd).

## Effect of *Pantoea* sp. ZNP-5 on plant growth under Cu stress

After growth for 21 days, significant differences in plant biomass were in different treatments. The addition of a different level of Cu (100–300 mg $kg^{-1}$) metal inhibited the plant biomass. However, the addition of bacterial inoculum ZNP-5 significantly ($p = 0.05$) improved the biomass. The increase in biomass regarding fresh weight was 34, 59, 74, and 51% at 0, 100, 200, and 300 mg $kg^{-1}$, respectively (Table 1). The results demonstrated that the addition of strain to the soil increased the dry biomass in the range of 27–80% under gradient (100–300 mg $kg^{-1}$ Cu) metal stress. Furthermore, the effect of Cu stress and a bacterial addition was also monitored on the chlorophyll a and b content. It is evident from Table 1 that increases in Cu level from 100 to 300 mg $kg^{-1}$soil, decreases the chlorophyll a and b in the range of 13–123% and 24–169%, respectively. The application of strain ZNP-5 significantly increased ($p = 0.05$) the chlorophyll a content of 41, 31, 98, and 64% at 0, 100, 200, and 300 mg $kg^{-1}$ of Cu in the soil as compared to respective control plants (Table 1). Similarly, the increase in chlorophyll b was 48, 47, 80, and 74% at 0, 100, 200, and 300 mg $kg^{-1}$ of Cu stress in bacteria-inoculated plants (Table 1). The effect of bacterial inoculation on shoot/root length has been summarized in Suppl. Table 2.

## Accumulation of Cu and distribution in plant

The bacterial treatment significantly improved the accumulation of Cu in the wheat plant tissue to the different degrees. As seen in Table 2, the accumulation of Cu was more in the shoots as compared to roots. The observed results illustrated that bacterial application mobilizes the metal content in the shoot tissue as compared to root. There was no any significant change in the shoot-Cu content at 0 mg $kg^{-1}$ Cu. However, bacterial inoculation significantly improved the Cu content in shoot and root of wheat plant treated with other Cu concentrations (100, 200, and 300 mg $kg^{-1}$ Cu). The highest significant ($p = 0.05$) accumulation of Cu in the shoot was 87% at 100 mg $kg^{-1}$ Cu, followed by 73 and 57% at 300 mg $kg^{-1}$ Cu and 200 mg $kg^{-1}$ Cu, respectively. The accumulation of Cu in the roots of wheat plant increased in the range of 21 to 64% in bacterial-primed plants. The highest significant ($p = 0.05$) accumulation of Cu content in the root was 64% at 200 mg $kg^{-1}$ Cu, followed by 60, 56, and 21% at 300, 100, and 0 mg $kg^{-1}$Cu as compared to respective control plants (Tables 2 and 3).

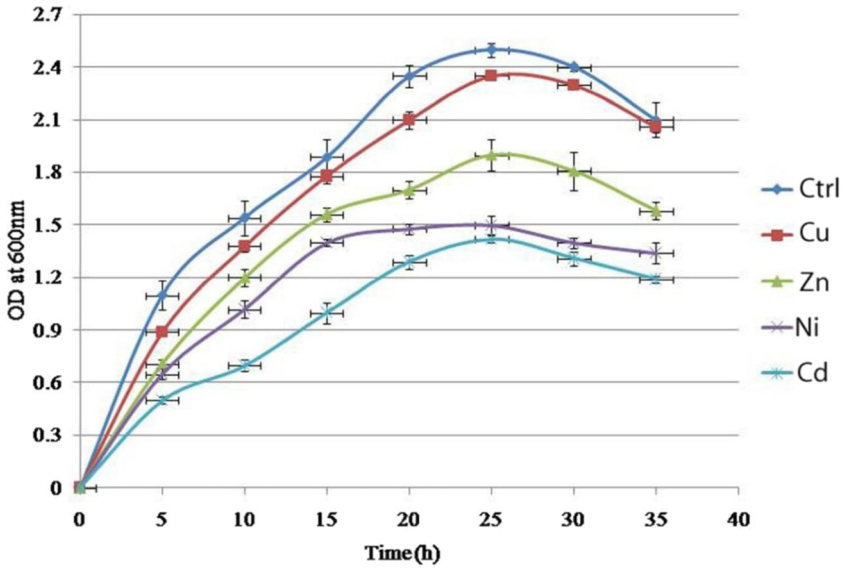


**Fig. 2** The growth pattern of test isolate *Pantoea* sp. ZNP-5 in metal supplemented medium. Error bars are ±standard deviation ($n = 3$)

## Antioxidant activities

The activity of various antioxidant enzymes in wheat plants was evaluated in the presence of bacterial inoculum to monitor the ROS level under metal stress. It can be seen from Fig. 3 that metal stress induced the production of tested antioxidant enzymes; however, its level was more prominent in the presence of bacterial inoculums. The ZNP-5 inoculation significantly ($p = 0.05$) increased the SOD activity by 26, 41, and 39% at 0, 100, and 200 mg $kg^{-1}$ of Cu stress, respectively, as compared to their control (Fig. 3a). However, there was no significant change in SOD activity at 300 mg $kg^{-1}$. Considering the peroxidase (POX) activity, it is evident from Fig. 3b that at 100 and 200 mg $kg^{-1}$, the increase in activity was 42 and 39% ($p = 0.05$) in ZNP-5-treated plants as compared to corresponding control. There was no significant change observed at 0 and 300 mg $kg^{-1}$. Similarly, the bacterial primed plants showed higher catalase activity as compared to control plants. The significant ($p = 0.05$) increase in catalase (CAT) activity was 30, 35, 28, and 25% at 0, 100, 200, and 300 mg $kg^{-1}$ (Fig. 3c).

**Table 1** Biochemical and physiological characteristic feature of strain ZNP-5

| Characteristic (s) | Activity | Carbohydrate | Activity |
|---|---|---|---|
| Gram reaction | – | Sodium gluconate | – |
| Catalase | + | Glycerol | + |
| Indole | – | Salicin | + |
| MR | – | Dulcitol | – |
| VP | + | Inositol | – |
| Amylase | – | Sorbitol | + |
| Lipase | + | Mannitol | + |
| Urease | + | Adonitol | + |
| Nitrate reductase | + | Arabitol | + |
| | | Erythritol | – |
| Carbon utilization test | | α-Methyl-D-glucoside | + |
| Carbohydrate | Activity | Rhamnose | + |
| Cellobiose | + | Melezitose | – |
| L-Arabinose | – | Inulin | + |
| Sucrose | + | Mannose | + |
| Lactose | + | α-Methyl-D-mannoside | – |
| Xylose | + | Xylitol | – |
| Maltose | + | ONPG | – |
| Fructose | + | Esculin hydrolysis | + |
| Dextrose | + | D-Arabinose | + |
| Galactose | + | Citrate utilization | – |
| Raffinose | + | Malonate utilization | – |
| Trehalose | + | Sorbose | – |
| Melibiose | – | | |

## Biochemical analysis

The change in certain compatible solutes namely proline, malondialdehyde (MDA), total soluble sugar (TSS), and total protein content (TPC) known to play important protective roles during stress condition. Differential productions of the above components were detected in bacterially treated and untreated plants under Cu stress condition. The proline content in ZNP-5 inoculated plants significantly ($p = 0.05$) enhanced by 35% as compared to a respective control without Cu stress. The significant ($p = 0.05$) increase in proline content (37%) was observed at 100 mg $kg^{-1}$of Cu, followed by 33 and 27% at 300 and 200 mg $kg^{-1}$ of Cu stress, when compared to un-inoculated control treated with respective metal stress (Fig. 4a).

**Table 2** Effect of ZNP-5 inoculation on different growth parameters of wheat plant grown under gradient Cu stress condition

| Treatment | Cu stress (mg/kg) | FW (g) | DW (g) | Chl a (mg $g^{-1}$ FW) | Chl b (mg $g^{-1}$ FW) |
|---|---|---|---|---|---|
| Control | 0 | 2.81 ± 0.87 | 0.280 ± 0.019 | 3.10 ± 0.57 | 1.75 ± 0.24 |
| Control | 100 | 2.15 ± 0.70 | 0.235 ± 0.017 | 2.75 ± 0.30 | 1.41 ± 0.15 |
| Control | 200 | 1.65 ± 0.69 | 0.178 ± 0.013 | 1.80 ± 0.41 | 0.91 ± 0.21 |
| Control | 300 | 1.48 ± 0.57 | 0.127 ± 0.019 | 1.39 ± 0.18 | 0.65 ± 0.10 |
| ZNP-5 | 0 | 3.78 ± 1.07* | 0.357 ± 0.039 | 4.38 ± 0.31* | 2.34 ± 0.37 |
| ZNP-5 | 100 | 3.35 ± 0.91* | 0.326 ± 0.021* | 3.61 ± 0.27* | 2.08 ± 0.19* |
| ZNP-5 | 200 | 2.87 ± 0.54* | 0.281 ± 0.027 | 3.58 ± 0.29* | 1.49 ± 0.17* |
| ZNP-5 | 300 | 2.10 ± 0.59* | 0.229 ± 0.031* | 2.29 ± 0.33* | 1.07 ± 0.12* |

Growth was measured at 21 days after seed germination under metal stress conditions

*FW* fresh weight, *DW* dry weight, *Chla* chlorophyll a, *Chlb* chlorophyll b

The values are mean ± SD of five measurements in triplicate sets ($n = 15$). Single asterisk (*) represents the significant difference from respective control according to Duncan's multiple range test ($p = 0.05$)

It is evident from Fig. 4b, which with the increase in Cu stress MDA content increased by 65%, however, bacterial application significantly ($p = 0.05$) decreased the MDA content by 49%. Also, MDA contents significantly decreased in the leaves of the wheat plant with different levels of Cu stress in ZNP-5-inoculated plants. The highest ($p = 0.05$) decrease of 41% was observed at 300 mg $kg^{-1}$, followed by 34, 30, and 28% at 200, 100, and 0 mg $kg^{-1}$, respectively, as compared to the corresponding control plants.

The results of total soluble sugar estimation revealed that bacterial inoculation significantly ($p = 0.05$) increased its content by 32, 43, and 36% at 100, 200, and 300 mg $kg^{-1}$ of Cu in soil (Fig. 4c). A significant difference was found for total protein content in wheat leaves stressed with different metal stress. From Fig. 4d, it can be seen that bacterial inoculation significantly ($p = 0.05$) increased the total protein content by 48, 46, and 25% at 100, 200, and 300 mg $kg^{-1}$ of Cu stress as compared to respective control plants.

## Oxidative stress measurement

Bacterial inoculation protected the wheat plants from the Cu-induced oxidative damages. It was monitored by evaluating the $H_2O_2$ and $O_2^-$ content as well as by measuring membrane stability index. The increase in the Cu content in the soil from 100 to 300 mg $kg^{-1}$ increased the $H_2O_2$ content from 29 to 107%. However, its content decreased significantly in the presence of isolate ZNP-5. The highest ($p = 0.05$) decrease with 42% was recorded at 200 mg $kg^{-1}$, followed by 30, 23, and 20% at 100, 0, and 300 mg $kg^{-1}$ of Cu, respectively, as compared to corresponding control (Fig. 5a). Decrease in the MSI was noted in the range of 22 to 110% under increased Cu stress; however, stability was significantly ($p = 0.05$) elevated to 50, 33, 16, and 15% at 200, 100, 0, and 300 mg $kg^{-1}$ of Cu stress, respectively, over the control (Fig. 5b). Exposure to Cu stress caused a substantial increase to the $O_2^-$ content in wheat plants. The effect was found to increase concerning the gradual increase of Cu content in the soil. However, a decrease in

**Table 3** The influence of bacterial strain on the mobilization of Cu in different tissue (shoot and root) of wheat plant

| Cu content (mg $kg^{-1}$ DW) | | | | |
|---|---|---|---|---|
| Stem | | | Root | |
| Treatment | Control | ZNP-5 treatments | Control | ZNP-5 treatments |
| (mg $kg^{-1}$) | | | | |
| 0 | 21.10 ± 3.40 | 23.48 ± 2.90 | 31.57 ± 3.87 | 38.19 ± 5.10 |
| 100 | 72.40 ± 6.10 ($p < 0.01$) | 135.70 ± 3.80** | 59.80 ± 1.78 ($p < 0.01$) | 93.45 ± 5.45** |
| 200 | 122.0 ± 7.47 ($p < 0.01$) | 191.8 ± 6.38** | 88.45 ± 4.30 ($p < 0.01$) | 145.40 ± 4.67** |
| 300 | 181.75 ± 6.30 ($p < 0.01$) | 314.90 ± 4.45** | 131.6 ± 5.31 ($p < 0.01$) | 210.8 ± 7.29* |

Values are the mean ± standard deviation of triplicate sets ($n = 3$). Double asterisk (**) represents significant difference than the corresponding control value as per Duncan's multiple range test ($p < 0.01$, $p < 0.05$)

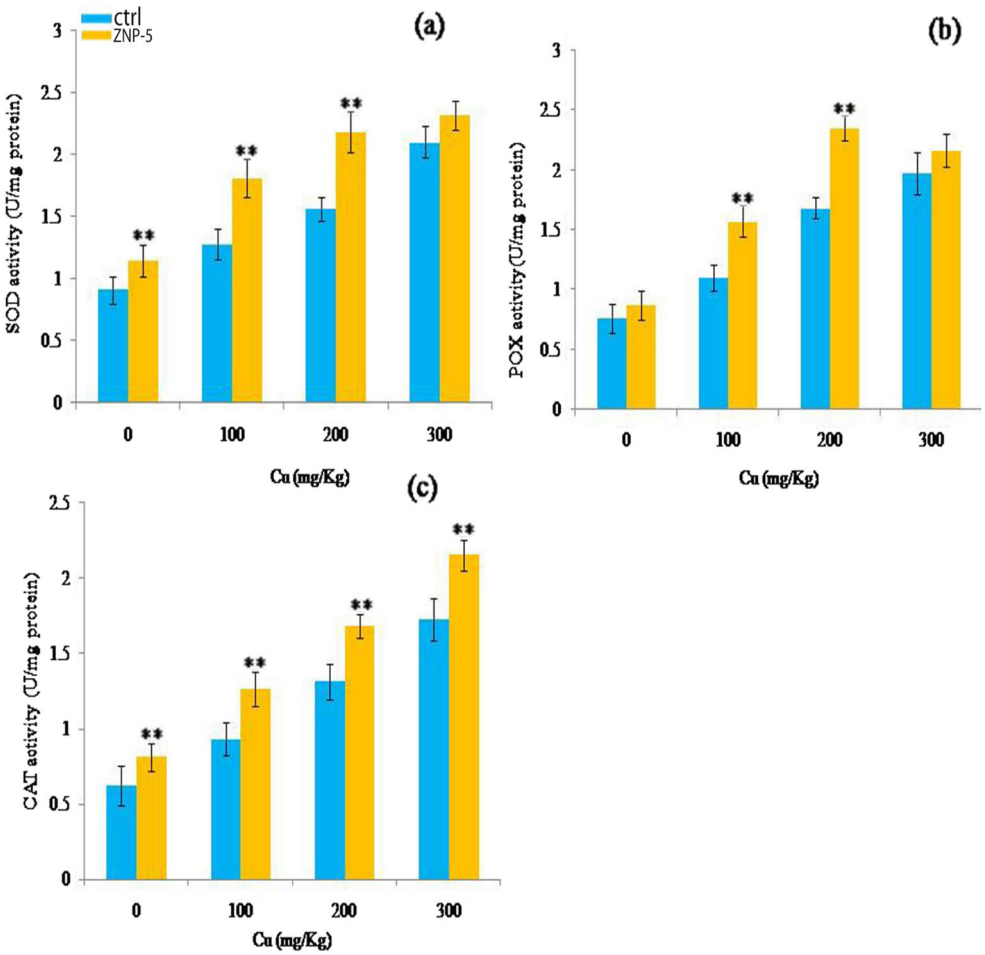


**Fig. 3** The effect of bacterial inoculation on the antioxidant enzymatic activities under metal stress. Values are the mean ± standard deviation ($n = 3$). Double asterisk (**) represents significant difference than the corresponding control value as per Duncan's multiple range test ($\leq 0.05$)

the $O_2^-$ content was noticed in response to bacterial inoculation. The highest ($p = 0.05$) decrease 52% was found to be at 200 mg $kg^{-1}$ Cu concentration, followed by 36, 24, and 21% at 100, 0, and 300 mg $kg^{-1}$ Cu concentration (Fig. 5c).

## Discussion

The present study aimed to explore a potent microbial strain that can alleviate the toxicity of heavy metal stress in host plants. The strain was specifically chosen for further studies due to its high metal solubilization ability in the soil. Previous studies (Jiang et al. 2008; Rajkumar et al. 2008) also demonstrated that metal mobilizing bacteria increases the water-soluble fraction of heavy metals. This behavior might be attributed to microbe-mediated alteration of soil pH, organic acid release, and oxidation/reduction reactions (Rajkumar et al. 2013). The present study is the first report of *Pantoea* sp. to protect the wheat plant from Cu-stress and its ability to alleviate the metal stress through polyphasic mechanisms.

It is evident from the previous studies that metal-resistant bacteria can enhance the metal uptake by the plants (De Souza et al. 1999; Whiting et al. 2001). Our results of experiments too demonstrated that the heavy metal-resistant bacterium ZNP-5 significantly enhanced Cu uptake by wheat plants. It was noticed that in the presence of ZNP-5, the copper accumulation in the roots and shoots of wheat was enhanced. However, the presence of bacterial isolate caused a lesser inhibitory effect on biomass production. The accumulation of metal (Cu) in plant tissues after ZNP-5 inoculation indicates its potential to tolerate, survive, and express plant beneficial traits under metal stress conditions. Although, role of bacteria in plant growth and heavy metal accumulation has been documented in several reports (Abou-Shanab et al. 2003; Idris et al. 2004; Sheng and Xia 2006), to the best of our knowledge, present study is the first to report role of metal-resistant PGPR *Pantoea* sp. in Cu mobilization with concurrent promotion of plant growth by plant growth study.

Cellular antioxidants play a vital role in protecting plants from the attack from metal stress-induced free radicals. ROS-scavenging enzymes including SOD, POX, and CAT belong to the first group of antioxidant enzymes which are involved in the defense system (Smeets et al. 2005). During exposure to Cu stress, there was no obvious variation in SOD, POX, and CAT activity. In contrary, application of bacterium increased the antioxidant activity in the Cu-stressed plants, indicating

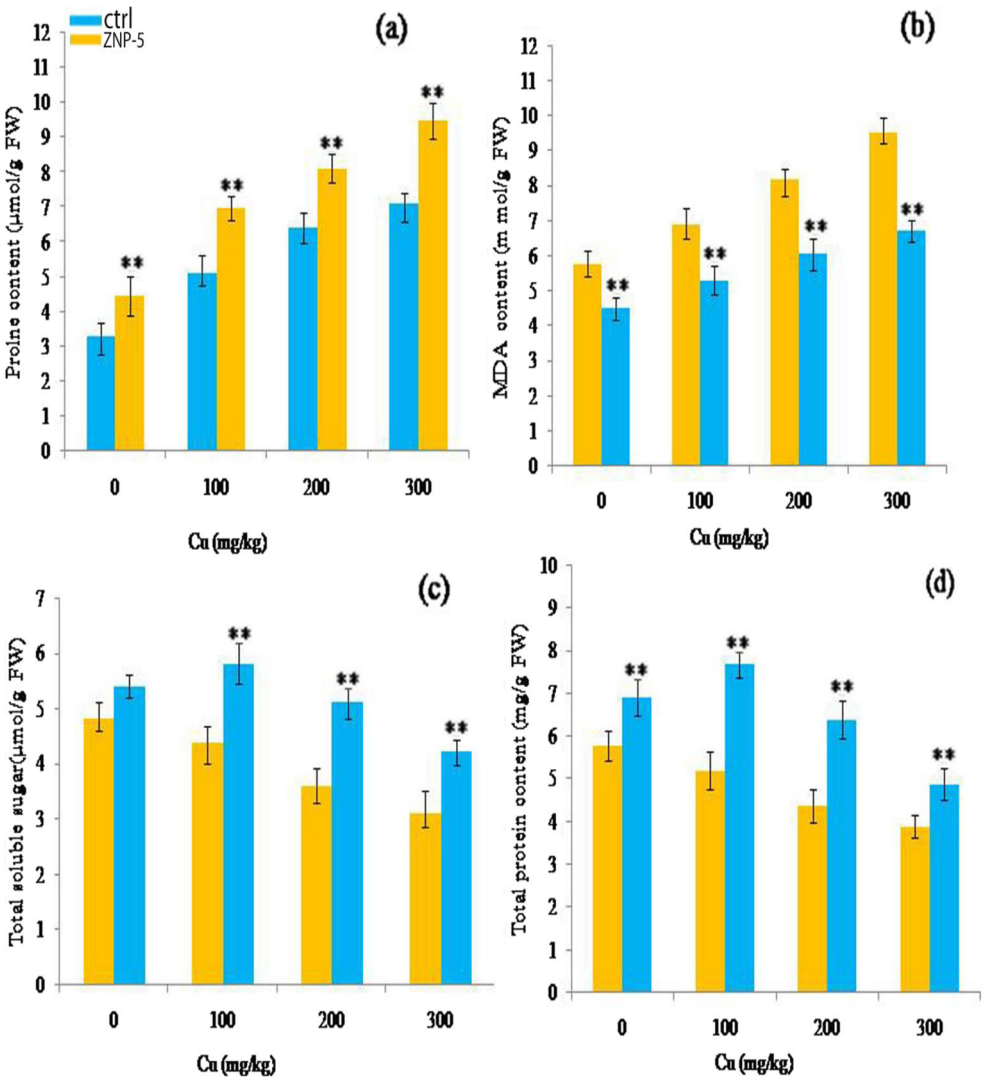


**Fig. 4** The effect of bacterial inoculation on various biochemical parameters of wheat plant under Cu stress. Values are the mean ± standard deviation ($n = 3$). Double asterisk (**) represents significant difference than the corresponding control value as per Duncan's multiple range test ($\leq 0.05$)

the modulation of the expression profile of metal-detoxifying enzymes to counteract the metal stress (Duponnois et al. 2006). The up-regulation of the activity of antioxidant enzymes depicts their role in detoxification of ROS. The enhanced activity of SOD suggests the efficient decomposition of $O_2^-$ into $H_2O_2$ at a very fast rate, thereby decreasing the risk of formation of hydroxyl free radicals. The increased activity of SOD could have resulted from the action of heavy metal ions and an in direct effect of increased level of superoxide radicals (Chongpraditnun et al. 1992). The induction of SOD encoding genes leading to the de novo synthesis of enzyme protein (Verma and Dubey 2003) might be associated with superoxide-mediated signal transduction (Fatima and Ahmad 2005). POX and CAT activity remove the $H_2O_2$ produced by dismutation activity of SOD. The higher POX and CAT activity could be responsible for quenching of $H_2O_2$ in metal-stressed conditions (Kaur et al. 2012).

Under heavy metal treatment, the accumulation of proline was decreased. However, its concentration was higher in ZNP-5-inoculated plants. The accumulation of proline suggests its role as an osmotic adjustment in Cu stress, which was also reported in earlier studies (Thounaojam et al. 2012; Yan and Tam 2013). Further, one of the most prominent symptoms of oxidative stress in the biological membrane is exhibited through lipid peroxidation, which can be observed from the increase in the MDA levels. We observed a statistically significant increase in MDA concentration for all the concentrations (100, 200, and 300 mg $kg^{-1}$ Cu) concerningcontrol. However, the level of MDA in the bacteria-treated plant was noted to decrease significantly ($p < 0.01$), which indicated the bacterial-mediated attenuated effect of metal stress on plants.

Soluble sugar and proteins, which includes a vast array of metabolic products, are widely known for their cellular metabolic stability. Accumulations of such essential metabolites are positively correlated with tolerance to oxidative stress in plants. In addition to directly scavenging ROS, soluble sugar also induces expression of genes encoding antioxidative enzymes (Sun et al. 2012). Cu stress can lead to the oxidative

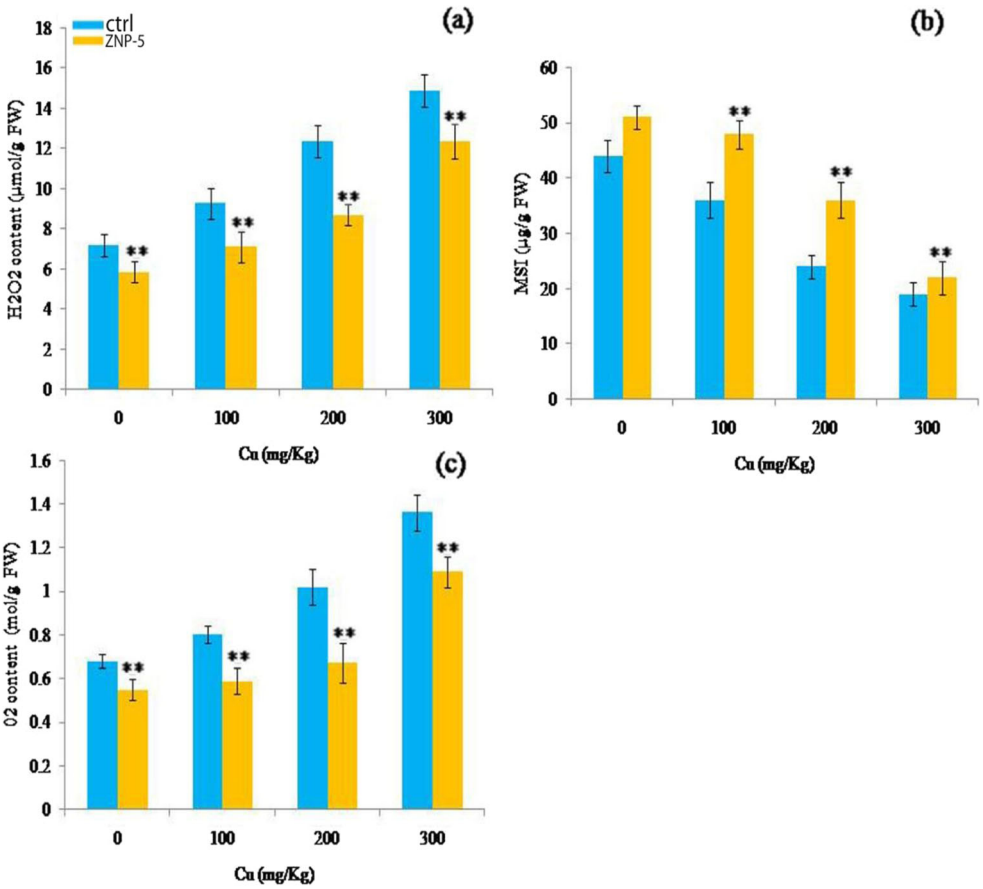


**Fig. 5** The evaluation of oxidative stress following bacterial inoculation under Cu stress. Values are the mean ± standard deviation ($n = 3$). Double asterisk (**) represents significant difference than the corresponding control value as per Duncan's multiple range test ($\leq 0.05$)

damage of molecular structure and enhances proteolytic activity which was evident from decreased protein content in Cu-stressed plants (Szollosi et al. 2012). It can be assumed that bacterial application serves as a stabilizer of protein and sugar structure and thus allows the wheat seedlings to maintain normal intracellular metabolism.

Exposure to heavy metals in soil enhances the content of stress markers such as $H_2O_2$, $O_2^-$ and lead to loss of membrane stability. This may be attributed to the increased acclimatization of Cu in plant tissues. In the previous studies, increased electrolyte leakage in wheat seedlings was observed on exposure to Ni and Pb stress (Gajewska et al. 2012; Kaur et al. 2012). This may be correlated with the increased accumulation of ROS that induces oxidative stress on exposure to metal stress. Oxidative stress leads to reduced metabolism and greater accumulation of $H_2O_2$ in plant tissues. On the other hand, bacterial inoculation gradually decreased the content of free radicals ($O_2^-$) hydrogen peroxide ($H_2O_2$) and maintained the membrane stability.

In the metal-stressed environment, rhizobacterium with PGP features could help to reduce copper stress and play an essential role in plant growth. The morphological, physiological, and functional traits of the selected bacterial isolate tested so that it can be utilized for bioremediation of Cu metal. Ability of the test isolate to utilize ACC as a nitrogen source owes due to the presence of ACC deaminase, which hydrolyzes ACC and enhances plant root and plant growth (Glick 2012). The test isolate was found to solubilize the inorganic phosphate which is one of the important attributes of plant growth-promoting bacteria to support plant growth (Jiang et al. 2008). The test isolate also exhibited production of siderophore which provides Fe nutrition, play important role in inhibiting pathogenic microorganisms, and help to reduce copper toxicity by increasing iron supply to the plant (Ali and Vidhale 2013). The overall plant growth-promoting attributes of the test isolate suggest its potential to alleviate copper toxicity and promote plant growth under copper stress.

There was significant plant growth promotion effect after bacterial inoculation in the Cu-contaminated soil. *Pantoea* sp. is well known for its plant growth-promoting features and improve crop yield in the field as well (De Maayer et al. 2014). Better plant growth following bacterial inoculation suggests the synergistic effect of plant growth-promoting features of the test isolate. In the previous study, it was observed that rhizobacterial inoculation significantly increases the metal tolerance in plants (Saleh and Saleh 2006). Similarly, the previous study has also indicated that inoculation with PGPR possessing the beneficial PGP traits enhances the growth of inoculated plants primarily through enhancing the nutrient uptake under heavy metal stress conditions (Ma et al. 2011).

A decrease in the level of chlorophyll a/b was observed in uninoculated Cu-stressed plants, which could be due to the inhibition of enzymes about Calvin cycles, peroxidation of thylakoid membrane, or substitution of $Mg^{2+}$ located in the tetrapyrrole ring of chlorophyll molecules (Yılmaz and Parlak 2011). Application of bacterial isolate increased the amount of the photosynthetic pigment, which suggested that wheat seedlings can tolerate Cu stress without notable leaf necrosis and chlorosis during the given time span. Furthermore, the colonization behavior should be tested in future studies under normal and stress condition to reveal the endophytic nature of the test isolate. Therefore, in the present study, microbial inoculant enhanced the capacity of plants to extract Cu from soil via the mechanisms discussed above, which are the increased heavy metal availability, increased plant tolerance, and growth and uptake.

## Conclusion

Plant-microbe interaction can be exploited to enhance phytoextraction efficiency of soil contaminated with heavy metal pollutants. The benefits of metal resistant and plant growth-promoting bacteria resulted in faster growth, better access to nutrients, and sustainable biomass production through enhanced remediation of metal-contaminated soils. In the present work, *Pantoea* sp. ZNP-5 was tested for the first time regarding its ability to alleviate the Cu-toxicity. Evoked antioxidative response (CAT, SOD, POX) following bacterial inoculation clearly revealed the effective plant mechanism to suppress ROS under high Cu concentrations. The stimulate production of various compatible solutes in response to the bacterium is likely to protect the plant from oxidative stress, induced by an elevated level of Cu in the soil. Thus, *Pantoea* sp. has emerged as a promising candidate for future to mitigate Cu from polluted soils. Further, the future studies are needed to screenout the genes responsible for metal tolerance and expression analysis in response to metal stress.

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