Role of Heavy Metal Resistant *Ochrobactrum* sp. and *Bacillus* spp. Strains in Bioremediation of a Rice Cultivar and Their PGPR Like Activities

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The present study demonstrates the metal toxicity ameliorating and growth promoting abilities of three different bacterial isolates when applied to rice as host plant. The three bacterial strains included a cadmium resistant *Ochrobactrum* sp., a lead resistant *Bacillus* sp. and an arsenic resistant *Bacillus* sp. designated as CdSP9, PbSP6, and AsSP9, respectively. When these isolates were used as inocula applied to metal-treated rice plants of variety Satabdi, the germination percentage, relative root elongation (RRE), amylase and protease activities were increased. The toxic effect of metal was reduced in presence of these bacteria. The overall biomass and root/shoot ratio were also enhanced by bacterial inoculation. Hydroponic studies showed that the superoxide dismutase (SOD) activity and malondialdehyde (MDA) level, which had been increased in the presence of metal stress in rice roots, were lowered by the bacterial inoculation. In addition, all three strains were 1-aminocyclopropane-1-carboxylate (ACC) deaminase and catalase positive, whereas siderophore producing ability was lacking in PbSP6. However, both PbSP6 and AsSP9 were protease positive and could hydrolyse starch. The data indicate that these bacteria have promise for bioremediation as well as for plant growth promotion.

Keywords: antioxidant, stress ethylene, metal tolerance, free-radicals, bioremediation, ACC deaminase, siderophores

Introduction

The release of heavy metals and metalloids from various sources such as metal industries, agrochemicals, and sewage sludge, is a major threat for the ecosystem, and human health. Once accumulated in the soil, the toxic metals inversely affect the composition of microbial populations, including the plant growth-promoting rhizobacteria (PGPR) and their metabolic activities. In an elevated concentration in the soil, metals are absorbed by roots and transported to different plant parts leading to impaired metabolism, reduced growth and decreased crop production (John *et al.*, 2009). Plants subjected to such metal toxicity also very often generate reactive oxygen species, which create oxidative stress, leading to increased levels of SOD and lipoxygenase activity (Baisak *et al.*, 1994). These enzymes are extremely harmful to plant cells and cause senescence. Two other common activities that lead to decreased plant growth and viability include - i) synthesis of stress ethylene and ii) decrease of iron sequestration. Under such conditions, plant growth promoting rhizobacteria (PGPR) play an important role in the rhizosphere. They can remove some of the metal toxicity to plants and improve plant growth and nutrition. They do so in two characteristic ways: first, they help to decrease the level of stress ethylene in plants growing in metal contaminated soil due to their 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. This results in the development of longer roots allowing the plants to establish themselves better during early stages of growth (Burd *et al.*, 1998; Glick *et al.*, 1998); and second, the PGPR release siderophores, iron complexing compounds that aid iron acquisition by plant roots in the hostile metal-polluted environments (Burd *et al.*, 1998, 2000; Glick *et al.*, 1998). Siderophores may also mobilize other heavy metals, increasing metal accumulation by resistant bacteria (Hu and Boyer, 1996). Such PGPR also stimulate seed germination, promote production of biomass and confer metal tolerance in plants (Glick, 2003). Although many soil bacteria are tolerant of metals and play important roles in mobilization and immobilization of these elements (Gadd, 1990), only limited attempts have been made to study their role in amelioration of toxic effects of metals on plants or their role as plant growth-promoting bacteria.

Phytoremediation is an established approach to remove contaminants from the environment. But using plants alone for bioremediation confronts many limitations. If these metal-accumulating plants are assisted by metal-resistant growth-promoting bacteria, the efficiency is increased several fold. Thus, in recent times, the application of PGPR has been extended to remediate contaminated soils in association with plant hyperaccumulators. This strategy of phytoremediation with appropriate heavy-metal-resistant rhizobacteria is gaining more attention worldwide.

The principal objective of the present study is to analyze the possible role of these bacterial isolates in conferring resistance to metals and metalloids on exposed cultivated rice

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plants. The metal-generated, toxicity-ameliorating effectiveness of these isolates on the germination and the early stage of seedling growth for the rice cultivar are examined in the present study. The PGPR-like phenotypes of the isolates were also analyzed.

Materials and Methods

Bacterial isolates and growth conditions

One Gram-negative, cadmium-resistant strain of *Ochrobactrum* sp. and two Gram-positive, lead- and arsenate-resistant strains of *Bacillus* spp., isolated from an old slag disposal site of IISCo, Burnpur, West Bengal, India were used in this study (Pandey and Maiti, 2008). They were identified on the basis of their phenotypic characteristics and 16S rDNA sequences. Their optimum growth conditions, and metal/metalloid accumulation efficiencies have already been determined (Pandey *et al.*, 2010, 2011). The GenBank Acession No. assigned to CdSP9, PbSP6 and AsSP9 are FJ798591, FJ798592 and FJ798593, respectively. The strains have been submitted in the MTCC (Microbial Type Culture Collection), IMTECH, Chandigarh, India.

The bacteria were cultivated in Luria Bertani medium (pH 7.5) aerobically at 37°C with 1% NaCl. The metal salts used were cadmium chloride, lead acetate and the metalloid salt used was sodium arsenate at a concentration of 50 μg/ml in each case. However, the minimum inhibitory concentration (MIC) for CdSP9 was 100 μg/ml, and that of PbSP6 and AsSP9 were 400 μg/ml and 180 μg/ml respectively (Pandey *et al.*, 2010, 2011). The exposure doses used in the enzymatic analyses and other assays were also kept at 50 μg/ml, because the toxicity of the three elements on the rice cultivar was not the same at any particular concentration.

Toxic effect of Cd, Pb, and As on germination and relative root elongation of the rice cultivar

The toxic effects of all three elements on germination percentage and relative root elongation (RRE) of *Oryza sativa* var. Satabdi were determined. Healthy seeds of this rice cultivar were supplied by the Chinsurah Rice Research Station, Chinsurah, Govt. of West Bengal, India. Seeds were surface sterilized by soaking them in 1.5% sodium hypochlorite for 15 min, then thoroughly rinsing them in sterile distilled water. Seeds were grown in Petri dishes on blotting papers imbibed with increasing concentrations (0, 10, 50, 100, 150, and 200 μg/ml) of cadmium chloride, lead acetate and sodium arsenate. The Petri dishes were then placed in darkness at 25°C for three days and transferred to a growth room (24°C±2°C, RH 70–80%, illuminated at 270 μE/m^2/sec) (Bhattacharya and Mukherjee, 2002). Germination percentage was measured against the control and the relative root rlongation was calculated after 10 days of growth, according to the formula: (Pandey *et al.*, 2007).

RRE (Relative Root Elongation) =

$$\frac{\text{Root elongation with heavy metal/metalloid}}{\text{Root elongation without heavy metal/metalloid}} \times 100$$

The concentration that inhibits germination and root growth by 50% was considered as the EC$_{50}$ for further analyses. The exposure doses used in the enzymatic analyses and other assays were kept at 50 μg/ml.

Influence of the bacterial inocula on germination and relative root elongation of the rice cultivar

Seeds were surface sterilized as described above and then transferred to Petri dishes with moist filter paper containing 50 μg/ml of each element and 2 ml bacterial inocula 1×10^8 cells/ml in 1× PBS (Phosphate Buffered Saline) were added aseptically to each Petri dish. The Petri dishes were placed in darkness at 25°C for three days and then transferred to a growth room (24°C±2°C, RH 70–80%, illuminated at 270 μE/m^2/sec). Germination percentage and RRE were determined after 10 days of germination.

Determination of amylase and protease activities in germinating rice

For amylase and protease activity, 72 h-incubated germinating seeds were extracted with 5 ml of chilled 0.1 M Na-phosphate buffer (pH 6.8) according to Snell and Snell (1971). Amylase and protease activities were then determined following the methods of Khan and Faust (1967) and Lowry *et al.* (1951) respectively. Activities of the enzymes were expressed according to Fick and Qualset (1975). The effect of resistant bacterial isolates was studied by inoculation of corresponding strains into 1× PBS, as mentioned above. Inoculation of *Escherichia coli* was the control.

Influence of the bacterial isolates on growth of *O. sativa* in hydroponic culture

Some freshly grown seedlings of uniform size were transferred to specially designed perforated plastic containers (Kachout *et al.*, 2009) containing modified half-strength sterile Hoagland's solution (Downs and Hellmers, 1975) having 50 μg/ml of each element, and 2 ml inocula (1×10^8 cells/ml in 1× PBS) of mid-log phase bacteria were then added. The seedlings were grown for a further three weeks and then biomass, root and shoot lengths, and SOD and MDA levels of the root were determined. Toxic effects of different concentrations of the three elements in question and the ameliorating effect of the respective resistant strains were noted in each case. The Hoagland's solution with the same metal and bacterial amendments were changed at three day intervals. Control sets received the same treatments except that no bacteria were added.

Measurement of SOD activity in root tissue of the rice cultivar

To determine SOD (SOD, EC 1.15.1.1) activity of the plants, 500 mg of root tissues were homogenized in 10 ml, prechilled, 100 mM Na-phosphate buffer (pH 6.8) containing 1% w/v PVPP (polyvinyl pyrolidone phosphate) and centrifuged at 4°C for 10 min. The supernatant was used as enzyme source and SOD activity was measured by its inhibition of the photoreduction of nitroblue tetrazolium (NBT) according to Dhindsa *et al.* (1981). The reaction mixture contained

1 M Na$_2$CO$_3$, 200 mM methionine, 2.25 mM NBT, 3 mM EDTA, 60 mM riboflavin, and 0.1 M phosphate buffer (pH 7.8). Riboflavin was added last. The test tubes were placed under a 40W fluorescent lamp at a distance of 30 cm at 25°C. After 30 min, the light was switched off and the absorbance was measured at 560 nm. The non-irradiated sample served as control. One unit of SOD denotes the amount of enzyme that inhibits the NBT photoreduction by 50%, and the enzyme was quantified on the basis of the inhibition percent.

Malondialdehyde (MDA) assay in root tissue of the rice cultivar

The MDA content was estimated using the procedure of Heath and Packer (1968). For this, 200 mg root tissue were homogenized in a 5 ml solution of 0.1% TCA (trichloroacetic acid), followed by centrifugation at 10,000 rpm for 5 min. To 1 ml of enzyme extract (supernatant), 3 ml of 5% TCA containing 1% TBA (Thiobarbituric acid) were added and the mixture was heated in a hot water bath at 95°C for 30 min. The reaction mix was then recentrifuged at 5,000 rpm for 5 min and the absorbance was measured at 532 and 600 nm in a UV-VIS spectrophotometer (Simadzu 190, Japan). The concentration was calculated from its extinction co-efficient of 155 μM^{-1} cm^{-1}.

ACC deaminase activity of the isolated strains

The 1-aminocyclopropane-1-carboxylate (ACC) deaminase activities of the isolated heavy metal resistant strains were qualitatively measured both in agar plate and in liquid culture. The ACC deaminase activity was determined by the method of Glick *et al.* (1995). For this, 1 μl of the pure bacterial culture was inoculated onto agar plates containing nitrogen free DF-medium modified by addition of ACC at 5.0 g/L. The ACC acted here as the sole nitrogen source. Plates were then incubated at 30°C and observed daily for colony formation for up to 4 days. Colonies produced were reinoculated in the same media and incubated under the same experimental conditions. The newly formed colonies in nitrogen-free modified DF-medium with addition of ACC were considered positive for ACC deaminase activity. Nitrogen free DF-medium without ACC supplement was used as the control, in which no colony appeared by 10 days. For further confirmation, a liquid culture study was performed. For this the bacteria were cultured first in rich liquid medium (TSB) and then transferred into DF-medium containing 3.0 mM ACC. The cultures were incubated on a rotary shaker at 30°C. DF medium without ACC or any other nitrogen source served as control. Growth is indicative of cells being ACC deaminase positive (Dell'Amico *et al.*, 2005). The absence of growth in nitrogen-free DF-medium confirmed the utilization of ACC as the source of nitrogen.

The quantitative estimation of ACC deaminase enzyme (EC 4.1.99.4) activity was made according to a modification of the method of Honma and Shimomura (1978), which measures the amount of α-ketobutyrate produced when the ACC deaminase cleaves ACC. The amount of α-ketobutyrate generated by this reaction was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate. One unit of ACC deaminase activity indicated the formation of 1 nmol of α-ketobutyrate/mg protein/h under these conditions.

Siderophore production and catalase, protease and starch hydrolyzing enzymes of the isolates

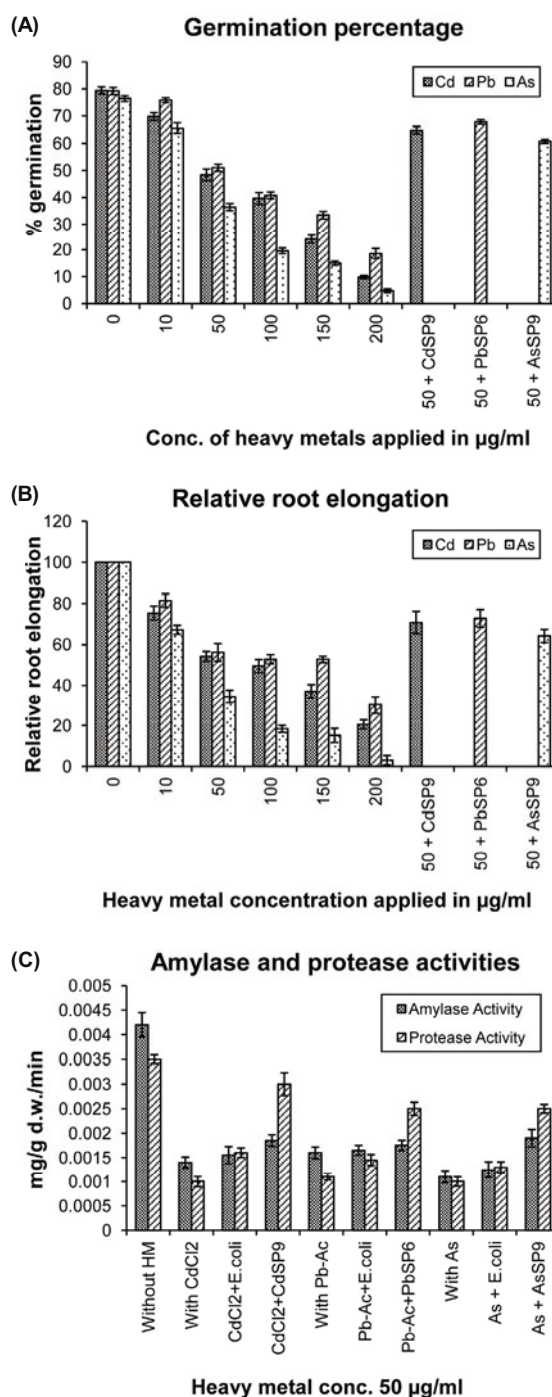Siderophore production was determined by the method of

Fig. 1. Toxic effects of metal salts (cadmium chloride, lead acetate and sodium arsenate) and the growth-promoting abilities of the strains CdSP9, PbSP6, and AsSP9, as measured in terms of germination percentage (A), relative root elongation (%) (B) and amylase and protease activities (C) on *Oryza sativa* variety *Satabdi*.

Schwyn and Neilands (1987). For this, 1 µl pure bacterial culture grown in LB was inoculated onto plates containing Chrome Azurol S (CAS) agar. Plates were incubated at 30°C and observed daily for orange color formation around each colony for up to 4 days. Experiments were performed in triplicate.

To determine the catalase activity of the isolates, the cultures were grown in a nutrient agar medium for 24 h at 30±2°C. The cultures were mixed with an appropriate amount of H_2O_2 on a glass slide to observe the evolution of oxygen.

Isolates were qualitatively screened for protease activity by spot inoculating them onto nutrient agar plates, pH 6.5,

containing 10% skimmed milk (w/v) and incubating for 48 h at 30°C. After the incubation period, the formation of a zone of clearance around the colony was taken as an indication of protease activity (Kanekar *et al.*, 2002).

To detect starch hydrolysis, the bacterial strains were streaked onto starch-agar plates and incubated at 30°C for 4 days. After the incubation, the plates were flooded with Lugol's iodine solution and a colorless zone around the streaks indicated a positive test for starch hydrolysis and the lack of a colorless zone around the streaks indicated a negative test.

The concentration of the protein extracts in all cases was determined by the Bradford method (Bradford, 1976).
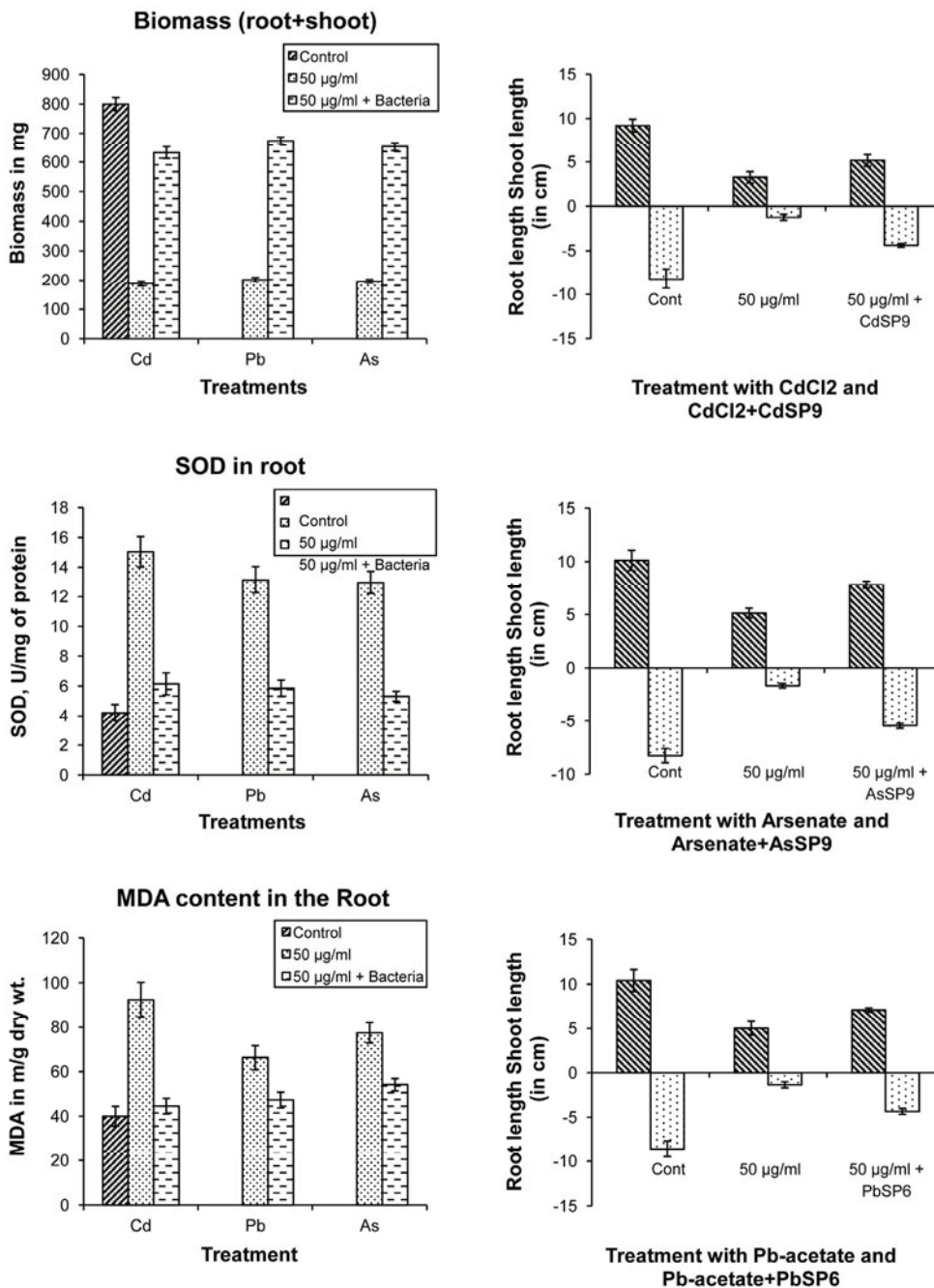
Fig. 2. Plant growth promoting abilities and phytotoxicity ameliorating activities of CdSP9, PbSP6, and AsSP9 in rice with respect to biomass, root/shoot ratio, SOD and MDA level.

Statistics

Values are the mean±SEM of 5 replicates. All data were subjected to Student's *t*-tests with a significance level of $P < 0.05$, using the SPSS software package.

Results

Amelioration of metal toxicity in the rice cultivar by bacterial inoculants

The plant growth promoting ability of the bacterial isolates is remarkable both with respect to enhancement of germination and also in increasing the biomass. Among the three elements used, arsenic was most toxic with respect to germination percentage and the Cd and Pb had a more or less similar inhibitory effect. With respect to relative root elongation also, arsenate was found to be most toxic. The exposure concentrations of the metals and metalloid used were 0, 10, 50, 100, and 200 µg/ml. The toxic effect of all three elements (Cd, Pb, and As) on the rice cultivar were variable, thus an exposure concentration of 50 µg/ml was selected for testing the effects of the isolates. When 50 µg/ml metal salts (cadmium chloride and lead acetate) and metalloid (sodium arsenate) were amended with the respective resistant bacterial inocula, the germination percentage was enhanced considerably in all three cases (Fig. 1A) and so was the relative root elongation (Fig. 1B). Amylase and protease enzymes are secreted during the time of germination and their activities could be an important factor for germinability. The activities of these enzymes were tremendously reduced in the presence of cadmium, lead and arsenate, with amylase being more affected than protease. Inoculation of metal or metalloid resistant bacterial isolates had a noticeable positive effect on the activity of protease, with CdSP9 being most effective. Amylase activity could not be restored to a higher level, except in case of AsSP9 (Fig. 1C).

Bacterial minimization of oxidative stress due to metal toxicity in the rice cultivar

In the hydroponic system, the bacterial inoculants had visible positive effects, yielding increased biomass and shoot and root lengths. The metals and the metalloid also induced superoxide radical generation in the rice plants, which increased SOD activity and MDA concentration. Addition of the bacterial isolates, however, ameliorated these toxicities almost to the control level. In this regard, the *Ochrobactrum* strain CdSP9 seemed to be more effective than the Gram-positive *Bacillus* strains PbSP6 and AsSP9 (Fig. 2).

Plant growth promoting activities of the isolated bacterial strains

All three heavy metal resistant bacteria had some important

PGPR traits (Table 1), two of which, ACC deaminase activity and catalase activity, were present in all of the strains. However, the ACC deaminase activity in the presence of metal was higher in the case of *Ochrobactrum* stain CdSP9 as compared to *Bacillus* strains PbSP6 and AsSP9, with the latter being the lowest. In the absence of metals, ACC deaminase activity was decreased in all cases, with CdSP9 giving a marked reduction in ACC expression. The siderophore-producing ability was also present in the Gram-negative *Ochrobactrum* sp. (CdSP9) and the Gram-positive *Bacillus* sp. resistant to arsenate (AsSP9); however, it was absent in lead resistant *Bacillus* sp. PbSP6. With respect to protease activity and hydrolysis of starch, both of the bacilli were positive; however, *Ochrobactrum* was negative.

Discussion

Plant growth promotion by rhizospheric bacteria has been observed by several authors (Glick *et al.*, 1998; Pishchik *et al.*, 2002; Wu *et al.*, 2006). Doelman (1985) reported that the efficiency of revegetation and phytoremediation of metal-contaminated sites was closely related to the presence of a higher concentration of metal-resistant microbial populations in the soil, which is likely to enhance nutritional assimilation and protective effects on plants. In the present study, we observed toxicity-ameliorating activities of the bacterial isolates that are resistant to cadmium, lead and arsenate. Germination percentage, relative root elongation, total biomass and root-shoot length, were positively influenced by the presence of the bacterial inoculants (Figs. 1 and 2). They also positively affected the activities of the enzymes amylase and protease, which are essential for seed germination (Fig. 1C). The presence of some PGPR-like traits (Table 1) at the same time is indicative of their involvement in plant growth promotion. This is in accordance with the growth-promoting abilities of both *Ochrobactrum intermedium* and *Bacillus cereus* with respect to chromium bioremediation in *Vigna radiata* (Faisal and Hasnain, 2006). The rhizospheric habitat of *Ochrobactrum* (*O. anthropi*), particularly in association to *Oryza sativa*, has been reported (Tripathi *et al.*, 2002). In addition, the role of *Pseudomonas fluorescens* and *P. tolaasii* in facilitating plant growth in the presence of cadmium in *Brassica napus* (Amico *et al.*, 2008) and that of Ni-resistant *Bacillus subtilis* in *Brassica juncea* has also been reported (Zaidi *et al.*, 2006). All these data suggest the relevance of the present study and are in accordance with the results reported here.

The inoculation of these metal- and metalloid-resistant bacteria into a system with the metal-treated rice host, helped to reduce the metal toxicity and thereby increased germination, overall biomass, amylase and protease activity (Fig. 1) and to reduce stress-induced enzyme activity (Fig. 2). The most plausible explanation for such an effect is that the ef-

Table 1. PGPR traits of the isolated heavy metal resistant strains

Strains	Siderophore production	ACC deaminase	Protease	Catalase production	Starch hydrolysis	Heavy metal tolerance
CdSP9	+	+	-	+	-	+
AsSP9	+	+	+	+	+	+
PbSP6	-	+	+	+	+	+
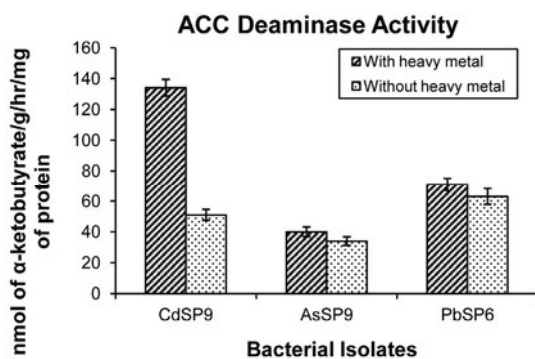
Fig. 3. ACC deaminase activity in CdSP9, AsSP9 and PbSP6 under metal/metalloid treated conditions (50 µg/ml each).

fective concentration of the metals and the metalloid is decreased due to their exclusion and immobilization by these bacteria, which indirectly removes the inhibitory effect of these metals or the metalloid (As) on germination, growth and enzymatic activities (Pishchik *et al.*, 2002). In addition to that, as these strains are ACC (1-aminocyclopropane-1-carboxylic acid) deaminase positive, another possible explanation could be the decrease in ethylene production due to the ACC deaminase activity, which consumes ACC, a precursor of ethylene biosynthesis (Glick *et al.*, 1998), in root tissue. Excess stress ethylene inhibits plant growth and development; hence, the decrease in its concentration enhances seed germination and seedling growth. Moreover, the cadmium resistant *Ochrobactrum* strain CdSP9 and arsenate resistant *Bacillus* strain AsSP9 excrete siderophores into the external environment. These are known to bind iron with high affinity so that plants are able to take up and utilize the iron taken from the environment by these complexes. Thus PGPR are able to protect plants against the inhibitory effect of a high concentration of metals by providing the plants with a sufficient amount of iron (Hu and Boyer, 1996). ACC deaminase and siderophore-producing bacteria can help plants to overcome many of the effects of high levels of metal contamination (Burd *et al.*, 1998, 2000). A direct relationship between ACC deaminase activity and plant root length promotion was established using ACC deaminase deficient mutants of *Pseudomonas putida* GR12-2 (Glick *et al.*, 1994) and *Enterobacter cloacae* UW4 (Li *et al.*, 2000). Belimov *et al.* (2005) reported Cd-resistant PGPR strains of *Variovorax paradoxus*, *Rhodococcus* sp., and *Flavobacterium* sp., which were capable of stimulating root elongation of *Brassica juncea* seedlings in the presence of toxic Cd concentrations. A positive correlation between the *in vitro* ACC deaminase activity of the bacteria and their stimulating effect on root elongation suggests that utilization of ACC is an important bacterial trait determining root growth promotion. The role of ACC deaminase in growth promotion is further evidenced in genetically modified *Pseudomonus aspleni* expressing ACC deaminase. This strain significantly stimulated seed germination in the presence of a high concentration of copper (Reed and Glick, 2005).

The SOD activity is remarkably reduced in the presence of the bacterial inoculants in all three cases indicating a re-

duced level of stress in the tissue of bacterially inoculated plants than in uninoculated, metal- or metalloid-treated tissue. Also, MDA (malondialdehyde), which is the last product of membrane lipid peroxidation, a process that injures plant cell membranes severely, and consequently disturbs photosynthesis and respiration of plants. The MDA content of plant tissue could be used to assess the degree of membrane-lipid peroxidation and its response to environmental stress (Chen, 1991). MDA decreases considerably in the bacterially inoculated experimental sets compared to the uninoculated sets. So, toxicity amelioration by neutralizing oxidative stress may be another possible mechanism by which these bacteria assist plant growth. The ability of plant growth-promoting bacteria to reduce metal ion phytotoxicity by sharing the metal load resulting from absorption and bioaccumulation has been reported (Zaidi *et al.*, 2006).

Conclusion

The bacterial strains used in this study were isolated from a slag disposal site of an iron and steel factory. The cadmium resistant CdSP9 was identified as *Ochrobactrum* sp. and the lead and arsenate resistant strains PbSP6 and AsSP9 belonged to the *Bacillus cereus* cluster. The present study establishes these strains as potential organisms for bioremediation of corresponding metal pollution. They are helpful in inducing germination, plant growth and reducing toxicity as they decrease the level of stress-induced SOD and malondialdehyde in the rice host. In addition, they also have ACC deaminase activity and several other PGPR traits, including siderophore production (CdSP9 and AsSP9).

Acknowledgements

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