

Role of Cadmium and Lead Tolerant *Pseudomonas aeruginosa* in Seedling Germination of Rice (*Oryza sativa* L.)

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Abstract

In the present study, cadmium and lead tolerant bacteria were isolated from contaminated crop field nearby industrial sites, garage and petrol pumps of Cachar District of Assam, India. Bacterial isolates were characterized on the basis of morphological, physiological, biochemical characteristics and 16S rDNA gene sequencing. In presence of cadmium, *Pseudomonas aeruginosa* SN1 and *Pseudomonas aeruginosa* SN3 showed the highest MIC for cadmium and lead. These two isolates were taken under consideration for pot experimental studies.

After 20 days of seedling inoculation in Cd treated soil and application of *P. aeruginosa* SN1, it has been observed that rice plant (*Oryza sativa* L.) attains 10.1% (at 20 mg/kg Cd) and 18.6% (at 50 mg/kg Cd) increased shoot growth as compared to control pots without bacterial inoculation. *P. aeruginosa* SN1 and *P. aeruginosa* SN3, showed significant result at 20 mg/kg of lead in soil but, failed to show any significant response at 50 mg/kg of lead in soil. Overall study demonstrated that *P. aeruginosa* SN1 and *P. aeruginosa* SN3 could remediate cadmium and lead contaminated soil at concentration below 20mg/kg, thus dedicating sites which are set aside for long term agricultural purpose.

Keywords: Minimum inhibitory concentration; *Oryza sativa* L.; Cadmium; Lead; *Pseudomonas aeruginosa*

Introduction

Excessive accumulation of heavy metals in agricultural soils through wastewater irrigation, results in soil contamination that lead to elevated heavy metal uptake by crops, and thus affect food quality and safety [1]. Heavy metal accumulation in soils and plants is of increasing concern because of the potential human health risks. Heavy metals are included in the main category of environmental pollutants as they can remain in the environment for long periods; their accumulation is potentially hazardous to humans, animals and plants [2].

Metals are directly and/or indirectly involved in all aspects of microbial growth, metabolism and differentiation [3]. Metals and their compounds interact with microbes in various ways depending on the metal species, organism and environment, while structural components and metabolic activity also influence metal speciation and therefore solubility, mobility, bioavailability and toxicity [3-6]. Many metals are essential for life, e.g. Na, K, Cu, Zn, Co, Ca, Mg, Mn and Fe, but all can exert toxicity when present above certain threshold concentrations. Other metals, e.g. Cs, Al, Cd, Hg and Pb, have no known essential metabolic functions but all can be accumulated. Microbes are intimately associated with the biogeochemical cycling of metals, and associated elements, where their activities can result in mobilization and immobilization depending on the mechanism involved and the microenvironment where the organism(s) are located [5-8].

Some reports have shown that indigenous microbes and plant-microbe symbionts tolerate high heavy metal concentrations in different ways and may play a significant role in the restoration of contaminated soil [9,10]. The objectives of the present study are as follows:

1. Isolation and identification of cadmium and lead tolerant bacteria from contaminated crop field
2. Pot experimental studies to evaluate the effectiveness of isolated strains in seedling germination of *Oryza sativa* at different

concentration of Cd and Pb in soil

Materials and Methods

Collection of soil sample and selective isolation of *Pseudomonas* spp.

Soil samples were first collected from contaminated crop fields nearby paper industry, garage and petrol pumps of Cachar District of Assam, India. Soil samples were collected in sterilized polythene bags and immediately brought to the laboratory. Selective isolation of *Pseudomonas* spp. was performed by spreading the samples on *Pseudomonas* Isolation Agar (PIA) media. Individual distinct colonies were further undergone repeated sub-culturing and were identified by their morphological and biochemical characteristics [11].

Identification by 16S rDNA sequencing

Genomic DNA was extracted from bacterial isolates as described by Sambrook et al., [12] and amplification of 16S rDNA gene was performed. The PCR procedure consisted of preparation of master mix for PCR containing 10X Taq buffer A (1 X), 10 mM MgCl₂ (1.5 µl), 10mM dNTP mix (1.0 µl) and sterile distilled water. The gene fragment was amplified using 20 ng of the DNA, 100 ng of each primer (forward and reverse) and Taq polymerase (3 U). The reaction volume was adjusted to 50 µl using sterile distilled water. The ~1.4 kb-PCR products of 16S rDNA genes were used for DNA sequencing. After sequencing,

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the ~1400 bp sequence were first analyzed by NCBI-BLAST for finding closest homologous sequence. The first ten homologous sequences were selected and were aligned by CLUSTAL X2. Finally, a phylogenetic tree was constructed by MEGA using Neighbour Joining method.

Minimum inhibitory concentration of cadmium of bacterial isolates

Minimal inhibitory concentrations (MICs) of cadmium and lead for isolated strains were determined by the plate dilution method [13]. MIC was determined against respective heavy metals Cd (CdCl_2) and Pb [$(\text{CH}_3\text{COO})_2\text{Pb}\cdot 3\text{H}_2\text{O}$] by gradually increasing the concentration on Nutrient Agar (NA) plates until the strains failed to give colonies on the plate. The initial concentration used was 50 $\mu\text{g/ml}$ and thereby the concentration was gradually increased by 10-15 $\mu\text{g/ml}$ each time on NA plates. The growth of cultures on last concentration was transferred to the higher concentration by streaking on the plate. MIC was recorded when the isolates failed to grow on plates.

Pot experimental studies

The bacteria showing the highest MIC were taken under consideration for the preparation of bacterial inoculum. The isolates were inoculated in nutrient broth and kept in shaker incubator at 120 rpm at $28 \pm 2^\circ\text{C}$ for 48 hours. After incubation period, 5 ml of broth was added to 45 ml distilled water for the formulation of bio-fertilizer and to carry out the pot experiment.

Seeds of *Oryza sativa* L. were collected from Krishi Vikas Kendra, Masimpur, Assam. The seed sizes and weights were homogenous. Clean seeds were dipped in water; floating seeds were discarded, while seeds settled on bottom of container were selected. Seeds were surface-sterilized with 95% alcohol for 30 seconds, followed by 0.1% (w/v) HgCl_2 for 1-2 min and then washed with sterile distilled water for 5-6 times [14]. The seeds were then put in a sterile petridish containing Hogland Solution and remain dipped overnight. The earthen pots (24 cm X 12 cm X 12 cm) were filled with sterilized sandy loam soil. Seeds were sown on all the pots to study the role of bacterial inoculation on seedling growth of *Oryza sativa*, sown in cadmium and lead incorporated soil. Pot experiments were performed in two different experimental groups and seedling growth of *Oryza sativa* were recorded after three weeks of exposure heavy metals (Cd and Pb) and bacterial inoculums.

After performing the pot experiment, SPSS 16.0 was used to analyze the statistical data. Descriptive statistics calculates the means of all replicates with standard error and deviations. Multiple comparison tests were performed to evaluate the effectiveness of each bacterial isolates. When analysis of variance (ANOVA) showed significant effects, Tukey's-b test (assuming equal variances) and Games-Howell test (assuming unequal variances) was done to make comparison between groups at $P < 0.05$ and $P < 0.01$.

Results and Discussions

Isolation and characterization of bacteria

Total viable counts ranges from 4.5×10^4 (CFU/g) in to 20×10^4 (CFU/g). At 1000 $\mu\text{g/ml}$ CdCl_2 concentration, resistance to cadmium varies from 48% to 79.2%. Samples collected from crop field nearby petrol pumps showed the highest frequency for lead tolerant bacteria, showed some resemblance with the work of Bruins et al. [15]. The lower values of microbial load at higher metal concentrations showed

correlation with the study of Anyanwu et al. [16].

All the isolates were identified based on their morphological and biochemical characterization [11]. The bacterial isolates identified in this study were mostly Gram-negative, the group that has been often found in metal polluted soils [17-20]. Among all the isolated strains, Ps-1 and Ps-4 showed the highest tolerance for Cd and Pb and hence selected for 16S rDNA sequencing. Neighbour-joining tree was constructed using both the sequences i.e., Ps-1 (Figure 1) and Ps-4 (Figure 2) and representative sequences from databases. It has been observed that the strain Ps-1 and Ps-4 had maximum sequence similarity with the species of *Pseudomonas aeruginosa* and occupied the same phylogenetic branch. The 16S rDNA gene sequences of the bacterial isolates were deposited in NCBI-GenBank having accession numbers: KF031122 (*Pseudomonas aeruginosa* SN1) and KF031123 (*Pseudomonas aeruginosa* SN3).

Screening for cadmium and lead tolerance

All the bacterial isolates exhibited high resistance to cadmium with minimum inhibitory concentration (MIC) ranging from 400 $\mu\text{g/ml}$ to 1800 $\mu\text{g/ml}$ (Table 1). In presence of cadmium, *P. aeruginosa* SN1 and SN3 (coded as Ps-1 and Ps-4 respectively) showed the MIC values as 1700 $\mu\text{g/ml}$ and 1800 $\mu\text{g/ml}$ respectively. *P. aeruginosa* SN3 exhibited highest MIC for lead as 170 $\mu\text{g/ml}$. The present study suggests that the microorganisms tolerant to metals appear to be the result of exposure to metal contaminated environment, which is fairly consistent with

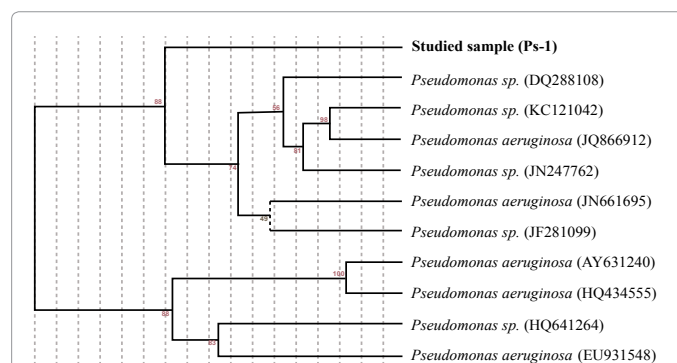


Figure 1: Phylogenetic relationship between studied sample (Ps-1) and representative species based on partial 16S rDNA sequences constructed using the neighbour-joining method.

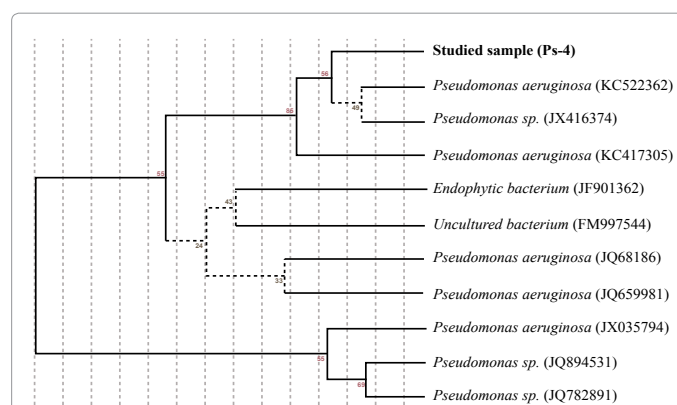


Figure 2: Phylogenetic relationship between studied sample (Ps-4) and representative species based on partial 16S rDNA sequences constructed using the neighbour-joining method.

Strain No	Minimum Inhibitory Concentration	
	Cadmium	Lead
Ps-1	1700 µg/ml	150 µg/ml
Ps-2	1100 µg/ml	80 µg/ml
Ps-3	800 µg/ml	100 µg/ml
Ps-4	1800 µg/ml	170 µg/ml
Ps-5	1000 µg/ml	150 µg/ml
Ps-6	1200 µg/ml	160 µg/ml
Ps-7	700 µg/ml	120 µg/ml
Ps-8	400 µg/ml	100 µg/ml

Table 1: Minimum inhibitory concentration of bacterial isolates to cadmium and lead.

Experimental setup	Cadmium concentration		Lead concentration	
	20 mg Cd/kg soil	50 mg Cd/kg soil	20 mg Pb/kg soil	50 mg Pb/kg soil
Control (without Cd and bacteria)	31.66 ± 0.42	31.66 ± 0.42	31.66 ± 0.42	31.66 ± 0.42
Uninoculated control (with HM only)	27.62 ± 0.35	25.18 ± 0.26	24.56 ± 0.32	23.66 ± 0.52
Inoculation of <i>P. aeruginosa</i> SN1	30.40 ± 0.30*	29.86 ± 1.33*	27.02 ± 0.19*	23.26 ± 0.36 ns
Inoculation of <i>P. aeruginosa</i> SN3	26.40 ± 0.86ns	22.24 ± 1.63ns	28.64 ± 0.32*	23.50 ± 0.29 ns

Values are mean ± standard deviation of five replicates; ns= non significant; *= significant at P<0.01; compared with uninoculated control; HM indicates respective heavy metals, i.e., cadmium and lead

Table 2: Effect of *Pseudomonas aeruginosa* on seedling growth and germination of *Oryza sativa* in cadmium and lead incorporated soil.

the findings of Ramteke [21].

Effect of *Pseudomonas aeruginosa* on seedling growth of *Oryza sativa* L. inoculated in cadmium incorporated soil

A large number of studies have been already carried out in different crop plants which indicate that, toxic levels of heavy metals affect structural and permeability properties of inner membranes and organelles, cause inhibition of enzymatic activities, nutrient imbalances, decreases in rates of photosynthesis and transpiration [22,23], stimulate formation of free radicals and reactive oxygen species resulting in oxidative stress [24], suppress seed germination and seedling growth [25], reproductive development, seed yield and seed quality [25] and induce deleterious anatomical and ultra-structural changes in crop plants [26,27].

In the present study, inoculation of *P. aeruginosa* SN1 significantly increases cadmium tolerance of *Oryza sativa* L. at all concentrations of Cd (20 mg/kg and 50 mg/kg) in soil. The increased seedling growth by *P. aeruginosa* SN1 having shoot length of 30.40 ± 0.30 cm at 50 mg/kg Cd in soil was further compared with shoot length of pots without Cd and bacterial inoculation (Table 2) and the result was found to be consistent with the work of Zhong et al. [28]. The statistical result thus obtained demonstrates *P. aeruginosa* SN1 as a potent isolate for bioremediation purpose whereas *P. aeruginosa* SN3 showed no significant increase in the seedling growth of rice plant (Table 2). Several studies have evidenced the fact that heavy metal-resistant and plant growth-promoting bacteria can protect plants from the toxic effects of metals [29-31].

After 20 days of seedling inoculation, two strains, *P. aeruginosa* SN1 and *P. aeruginosa* SN3, showed significant result at 20mg/kg of lead in soil. However, these isolates do not show any significant response at 50 mg/kg of lead in soil. Statistical analysis and multiple comparison studies highlight the fact that *P. aeruginosa* SN3 attains 14% increased

seedling growth as compared to uninoculated control pots at 20 mg/kg Pb in soil (Table 2). Present study shows some resemblance with the work of Vivas et al. [32], who found that the inoculation of *Trifolium repens* with *Brevibacillus* sp. B-I decreased the concentration of zinc in shoot tissues compared with respective uninoculated control. It can be inferred from the above experiment that, metal binding bacteria can reduce the metal bioavailability and restricts its entry into the plant root/shoot Madhaiyan et al. [33].

Conclusion

Present study demonstrated that *P. aeruginosa* SN1 could increase the growth of *Oryza sativa* L. in cadmium contaminated field. Both the tested *Pseudomonas aeruginosa* isolates showed significant result at 20 mg/kg of lead in soil, but the result was not significant at higher concentration of cadmium in soil. Overall study demonstrated that *P. aeruginosa* SN1 and *P. aeruginosa* SN3 could remediate cadmium and lead contaminated soil at concentration below 20mg/kg, thus dedicating sites which are set aside for long term agricultural purpose.

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