

***Pseudomonas aeruginosa* SN4 Enhances Seedling Growth of *Oryza sativa* in Cadmium Contaminated Soil**

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ABSTRACT

Pseudomonas spp. were isolated from different locations of Ponta Sahib, Himachal Pradesh, India and were characterized on the basis of morphological, biochemical and 16S rRNA gene sequencing analysis. Minimal inhibitory concentrations of Cd²⁺ for all *Pseudomonas* isolates were detected and *P. aeruginosa* SN4 exhibited highest cadmium tolerance upto 1800µg/ml. Most of the isolated strains showed multi-metal and multi antibiotic tolerance. In pot experimental studies, application of *P. aeruginosa* SN4 showed a remarkable increase in shoot length when sown in Cd incorporated soil (at 50 mg/kg). After 20 days of seedling inoculation in Cd incorporated soil and application of *P. aeruginosa* SN4, it has been observed that rice plant attains 12% increased seedling growth and germination as compared to uninoculated control pots. Overall study demonstrated that *P. aeruginosa* SN4 could increase the growth of *Oryza sativa* in cadmium incorporated soil, thus dedicating the sites which are set aside for long term agricultural purpose.

Key words: Minimum inhibitory concentration, Cadmium, Tolerance, *Oryza sativa*, *Pseudomonas aeruginosa* SN4

INTRODUCTION

Pseudomonas is a genus of gram negative, nonspore forming, rod-shaped bacteria. They are commonly found in soil, water and decaying matter and including some species that are plant and animal pathogens. *Pseudomonas aeruginosa* is a typical example of double assessment; in a clinical environment, it is one of the most significant opportunistic pathogenic bacteria and is responsible for the majority of nosocomial infections¹. However, in the field of environmental protection, the human health concerns of this organism are not recognized, and the strains of this species are commonly used for bioremediation purposes.

Pseudomonas spp. have been employed efficiently as biocontrol agents and some commercial products in the market has already shown their

efficacy, nevertheless, the applications of purified siderophores, as bacteriostatic or fungistatic agents in combination with other antibacterial factors will certainly raise a great interest. *Pseudomonas* spp. produces numerous compounds which are responsible for disease control and also helps in the growth of plants. These inhibitory compounds are siderophores, HCN, degradative extracellular enzymes such as chitinase, protease, cellulose, α-1,3glucanase and antibiotics such as pyrrolnitrin, pyoluteorin and phenazine^{2,3,4}.

MATERIALS AND METHODS

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

Soil samples were first collected randomly from seven different locations of Ponta Sahib, Himachal Pradesh, India. Sampling was done from

garages, welding shops, petrol pumps and other polluted sites. Samples were collected in sterilized polythene bags after proper labeling and immediately brought to the laboratory. Soil plating was done within 72 hrs. of sample collection. Selective isolation of *Pseudomonas* spp. was done by spreading the samples on Pseudomonas Isolation Agar (PIA) media and incubating at 37°C for 24 hrs. Pure cultures were finally obtained by repeated sub-culturing followed by cadmium tolerance tests.

Characterization of the bacterial isolates

Pure culture of *Pseudomonas* spp. that were tolerant to cadmium were identified up to species level by their morphological and biochemical characterization^{5,6} and finally confirmed by 16S rDNA sequencing.

Identification by 16S rDNA sequencing

Pure cultures were grown until log phase and genomic DNA was extracted from bacterial isolates⁷. The amplification of 16S rRNA gene was done by using forward primer and reverse primer. The ~1.4 kb-PCR products of 16S rRNA genes were used for DNA sequencing. After sequencing, the sequence was analysed by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Ribosomal Database Project (<http://rdp.cme.msu.edu/>) for finding the closest homologous sequence. The first ten homologous sequences were selected based on their maximum identity score. The sequences were then aligned and a distance matrix was constructed followed by the construction of a phylogenetic tree by Neighbour-Joining method.

Screening for cadmium tolerance

All the isolates were checked for metal tolerance. Minimum Inhibitory Concentration (MIC) was determined against cadmium (CdCl₂) by gradually increasing the concentration of the cadmium on Nutrient Agar (NA) plates until the strains failed to give colonies on the plate. The initial concentration was 50 µg/ml and the cultures grown 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. Minimum inhibitory concentration for copper, lead and zinc were also determined.

Antibiotic sensitivity and resistance pattern of the recovered isolates

The isolates were tested for antibiotic sensitivity according to Kirby-Bauer disc diffusion method⁸ and the antibiotics discs were procured from 'HIMEDIA'. The antibiotics that were used in the present study are Amikacin, Amoxycillin, Ampicillin, Cefalexin, Cefixime, Ceftriaxone, Chloramphenicol, Gentamicin, Kanamycin, Methicillin, Ofloxacin and Tetracycline. The diameter of the inhibition zones was measured to the nearest mm and the isolates were classified as resistant (R), intermediate (I) and susceptible (S) following the standard antibiotic disk sensitivity testing method.

Pot experiment

Preparation of bacterial inoculum

Pseudomonas aeruginosa SN4, which showed a greater degree of tolerance for cadmium, was taken under consideration for the preparation of bacterial inoculum. The isolate was inoculated in nutrient broth and kept in shaker incubator at 120 rpm at 28 ± 2°C for 48 hours. After incubation period, 5 ml of broth was added to 45 ml distilled water for the formulation of biofertilizer and to carry out the pot experiment.

Collection and pre-sowing treatment of *Oryza sativa* seedlings

Seeds of *Oryza sativa* were collected from KrishiVikas Kendra, Masimpur, Assam. The size and weight of seeds 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) HgCl₂ for 1–2 min and then washed with sterile distilled water for 5–6 times⁹. The seeds were then put in a sterile petridish containing Hogland Solution and kept overnight.

Pot trial studies

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 *P. aeruginosa* SN4 on shoot growth of *Oryza sativa* sown in cadmium incorporated soil (at 20 mg/kg and 50 mg/kg of soil). Pot experiment was performed by adding the biofertilizer formulation to cadmium contaminated

soil. Seedling germination and its growth pattern was recorded everyday for three weeks.

Statistical analysis

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

Characterization of selected isolates

A total of thirty two *Pseudomonas* spp. were isolated from Ponta Sahib, Himachal Pradesh, India. Total viable counts ranges from 32×10^4 (CFU/g) to 43×10^4 (CFU/g). All the isolates were gram negative rod. Most of the isolates were found to be indole

Table. 1: Minimum inhibitory concentration of all the isolated strains

Isolate code	Minimum Inhibitory Concentration			
	Cadmium	Lead	Copper	Zinc
Ps-1	1400 µg/ml	150 µg/ml	50 µg/ml	1800 µg/ml
Ps-2	1100 µg/ml	80 µg/ml	60 µg/ml	1500 µg/ml
Ps-3	800 µg/ml	100 µg/ml	40 µg/ml	1600 µg/ml
Ps-4	700 µg/ml	110 µg/ml	30 µg/ml	1100 µg/ml
Ps-5	1000 µg/ml	150 µg/ml	40 µg/ml	1600 µg/ml
Ps-6	1200 µg/ml	160 µg/ml	50 µg/ml	1700 µg/ml
Ps-7	700 µg/ml	120 µg/ml	50 µg/ml	1200 µg/ml
Ps-8	400 µg/ml	100 µg/ml	30 µg/ml	1000 µg/ml
Ps-9	1100 µg/ml	140 µg/ml	20 µg/ml	1200 µg/ml
Ps-10	1000 µg/ml	150 µg/ml	50 µg/ml	1300 µg/ml
Ps-11	1800 µg/ml	170 µg/ml	60 µg/ml	1800 µg/ml
Ps-12	600 µg/ml	100 µg/ml	60 µg/ml	1000 µg/ml
Ps-13	1700 µg/ml	160 µg/ml	60 µg/ml	1600 µg/ml
Ps-17	1400 µg/ml	170 µg/ml	60 µg/ml	1800 µg/ml
Ps-15	300 µg/ml	100 µg/ml	30 µg/ml	1000 µg/ml
Ps-16	500 µg/ml	120 µg/ml	20 µg/ml	900 µg/ml
Ps-17	1200 µg/ml	170 µg/ml	60 µg/ml	1300 µg/ml
Ps-18	800 µg/ml	140 µg/ml	50 µg/ml	1000 µg/ml
Ps-19	800 µg/ml	150 µg/ml	40 µg/ml	1300 µg/ml
Ps-20	600 µg/ml	120 µg/ml	30 µg/ml	900 µg/ml
Ps-21	1200 µg/ml	150 µg/ml	60 µg/ml	1400 µg/ml
Ps-22	600 µg/ml	120 µg/ml	50 µg/ml	1300 µg/ml
Ps-23	400 µg/ml	120 µg/ml	40 µg/ml	1300 µg/ml
Ps-24	400 µg/ml	120 µg/ml	40 µg/ml	1000 µg/ml
Ps-25	1500 µg/ml	130 µg/ml	40 µg/ml	1200 µg/ml
Ps-26	1200 µg/ml	120 µg/ml	50 µg/ml	900 µg/ml
Ps-27	1800 µg/ml	170 µg/ml	60 µg/ml	1800 µg/ml
Ps-28	1000 µg/ml	130 µg/ml	40 µg/ml	1500 µg/ml
Ps-29	1200 µg/ml	120 µg/ml	30 µg/ml	1200 µg/ml
Ps-30	600 µg/ml	120 µg/ml	30 µg/ml	900 µg/ml
Ps-31	300 µg/ml	100 µg/ml	30 µg/ml	1000 µg/ml
Ps-32	1000 µg/ml	130 µg/ml	40 µg/ml	1500 µg/ml

negative, MR negative, VP negative and urease negative. Oxidase tests for *Pseudomonas* isolates were positive indicating them to be aerobic strains. Positive result for citrate test was observed, infers the ability of these organisms to utilize citrate as the sole source of carbon and energy.

Isolates were also able to produce an enzyme "nitrate reductase" resulting in the reduction of nitrate (NO₃).

The isolate which showed highest tolerance for cadmium and also showed significant result in pot experiment was selected for 16S rDNA sequencing. A neighbour-joining tree was generated using the sequence from Ps-11 (1393 bp) and representative sequences from databases. It has been observed that the strain code Ps-11 had maximum sequence

similarity with the species of *Pseudomonas aeruginosa* and occupied the same phylogenetic branch (figure 1). The sequence has been identified as *Pseudomonas aeruginosa* SN4 and submitted to NCBI-GenBank. The accession number thus obtained is KF447770.

Minimum Inhibitory Concentration (MIC) of the recovered isolates

All the bacterial isolates exhibited resistance to cadmium and the minimum inhibitory concentration (MIC) ranges from 300 µg/ml to 1800 µg/ml. There is a great variation in the tolerance capacity of isolated bacterial strains against cadmium chloride. Around 53% of the tested isolates were tolerant at 1000 µg/ml CdCl₂ concentration. *Pseudomonas aeruginosa* SN4 (Isolate code: Ps-11) exhibited high resistance with MIC for cadmium as 1800 µg/ml. Multi-metal

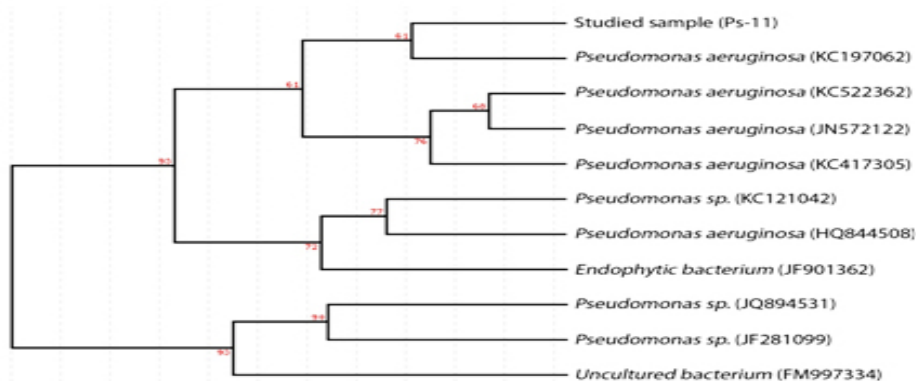


Fig. 1: Phylogenetic relationship between studied sample (Ps-11) and representative species based on partial 16S rDNA sequences constructed using the neighbour-joining method. Studied sample (Ps-11) has been submitted to NCBI-Genbank and the accession number thus obtained is KF447770 (*Pseudomonas aeruginosa* strain SN4)

Table. 2: Seedling growth of *Oryza sativa* inoculated with cadmium resistant *P. aeruginosa* SN4 in cadmium incorporated soil

Experimental setup	Shoot length (in cm)	
	Cd at 20mg/kg soil	Cd at 50mg/kg soil
Control (without Cd and <i>P. aeruginosa</i> SN4)	31.66 ± 0.42	31.66 ± 0.42
Uninoculated control (with Cd only)	27.62 ± 0.35	25.18 ± 0.26
<i>P. aeruginosa</i> SN4 + Cd	28.58 ± 0.98 ^{ns}	28.18 ± 0.49*

Values are mean ± standard deviation of five replicates; ns= non significant; *= significant at P<0.01; compared with uninoculated control

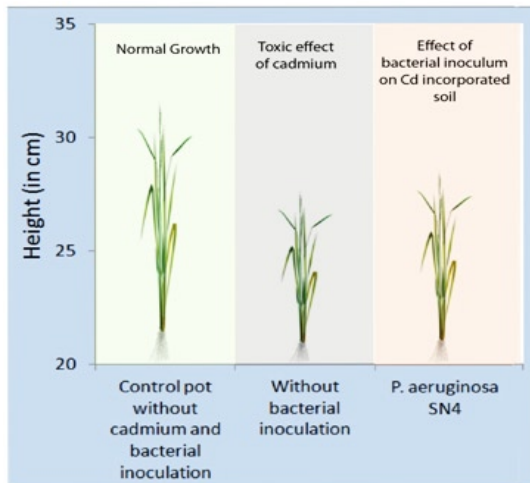


Fig. 2: Effect of *P. aeruginosa* SN4 on seedling growth of *Oryza sativa* inoculated in 50 mg/kg cadmium in soil and compared them with control sets

tolerance test showed that the MIC value for *P. aeruginosa* SN4 for lead was 170 µg/ml, copper was 60 µg/ml and zinc was 1800 µg/ml (table 1).

Antibiotic sensitivity and resistance pattern of *Pseudomonas aeruginosa* SN4

Most of the *Pseudomonas* spp. isolated in the present study exhibit high resistance pattern towards a group of antibiotics. It has been observed that *P. aeruginosa* SN4 were resistant to amoxicillin, ampicillin, cefalexin, cefixime, kanamycin, methicillin and tetracycline. This fact was also established by other researchers that multiple metal resistance bacterial isolates exhibit high resistance towards a group of antibiotics¹⁰.

Effect of *Pseudomonas aeruginosa* SN4 on growth of *Oryza sativa* inoculated in cadmium incorporated soil

Inoculation with *P. aeruginosa* SN4 significantly increases seedling germination and growth of *Oryza sativa* when compared with control sets. After 20 days of seedling germination, multiple comparison result reveals that *P. aeruginosa* SN4 attains 12% increased seedling growth as compared to uninoculated control pots at 50 mg/kg concentrations of cadmium in soil (table 2). However, at 20 mg/kg cadmium in soil, a slight increased germination (mean difference = 0.96 ± 0.63 cm) was observed which was statistically non-significant. The

result demonstrates the maximum bio-absorption of cadmium by *P. aeruginosa* SN4 at elevated levels of cadmium in soil.

DISCUSSION

Increased concern over the impact of cadmium on our environment, especially the vegetation has resulted in the increased interest in bioremediation strategies. Microscopic evidence showed that isolated bacteria were gram-negative, rod-shaped. Further, biochemical tests and 16S rDNA confirms the isolates as *Pseudomonas aeruginosa*.

A decrease in growth (cfu/g) of bacterial colonies was observed on increasing the cadmium concentration on culture plates at any given time interval compared to the control without metal amendment. The lower values of microbial load at higher metal concentrations showed correlation with the study of Anyanwu *et al.*¹¹. Present study demonstrated the cadmium resistance pattern of *Pseudomonas* isolates. Based on the MIC values and antibiogram pattern of the isolated strains and as studied by Bruins *et al.*,¹² *Pseudomonas* sp. shows resistance to a variety of toxic substances, heavy metal and antibiotics. *Pseudomonas aeruginosa* SN4 can tolerate upto 1800 µg/ml cadmium and also showed multi-metal tolerant activity.

The toxic levels of heavy metals effect structural and permeability properties of inner membranes and organelles, that results in inhibition of enzymatic activities, nutrient imbalances, decreases in rates of photosynthesis and transpiration^{13,14}, stimulate formation of free radicals and reactive oxygen species resulting in oxidative stress¹⁵, suppress seed germination and seedling growth, reproductive development, seed yield and seed quality¹⁶ and induce deleterious anatomical and ultra-structural changes in crop plants^{17,18}. In the present study, application of *Pseudomonas aeruginosa* SN4 showed a remarkable increase in seedling growth and germination when sown in cadmium incorporated soil (at both 20 and 50 mg/kg). After 20 days of seedling inoculation in Cd incorporated soil and application *P. aeruginosa* SN4, it has been observed that rice plant attains a significant seedling growth as compared to uninoculated control pots. Several studies have

evidenced the fact that cadmium-resistant and plant growth-promoting bacteria can protect plants from the toxic effects of metals^{19,20,21}. Overall experiment demonstrated that *P. aeruginosa* SN4 (Genbank Acc. No: KF447770) could increase the growth of *Oryza sativa* in cadmium contaminated crop field (figure 2), thus dedicating sites which are set aside for long term agricultural purpose. Further research is still required to expand the knowledge of the *P. aeruginosa* SN4 before being used for commercial purpose

However, in the second pot, cadmium was added at concentration 50 mg/kg in soil; demonstrates the toxic effect of cadmium in seedling germination and growth. In the final test pot, addition of *Pseudomonas aeruginosa* strain SN4 results in attaining a significant seedling growth as illustrated in table 2.

CONCLUSION

Present study isolated 32 cadmium tolerant bacteria from contaminated sites of Ponta Sahib, Himachal Pradesh, India. Most of the isolates exhibited multi-metal tolerance and were resistant to a group of antibiotics. *P. aeruginosa* SN4 showed

the highest tolerance for cadmium and was taken under consideration for pot experimental studies. Compared with control treatments, inoculation with *P. aeruginosa* SN4 influences the seedling germination at 50 mg/kg cadmium concentration in soil. It has been observed that, microbial inoculation altered the bioavailability of metals in soil, resulting in 12% increased seedling growth after 20 days of bacterial inoculation. It is evident from the present study that the application of HMRB specifically adapted to high concentrations of heavy metals will increase the ability to remediate heavy metal contaminated soils. Further research is still required to evaluate the bio-absorption potential of *P. aeruginosa* SN4 by field trials and the underlying mechanism of action.

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