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Siderophore Producing *Proteus vulgaris* KNP3 that Enhance Growth of Soybean Plant in Lead Contaminated Soil

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ABSTRACT

Heavy metals, being phytotoxic, cause growth inhibition and even plant death. Siderophore producing bacterial strain KNP3, isolated from the soil of a thermal power plant was found to be heavy metal resistant as well as plant growth promoting. It caused significant increase (P>5%) in root and shoot length of soybean (*Glycine max*) in presence of 660 µM lead acetate. Moreover, concentration of accumulated lead in soybean shoots and root were decreased in the presence of this isolate. Loss in chlorophyll content due to presence of lead could be overcome in the presence of bioinoculant (KNP3), which was comparable to plants grown in uncontaminated soil. 16s ribosomal DNA (16s r DNA) sequencing identified KNP3 as a strain of *Proteus vulgaris*.

***Key words*:** *Proteus vulgaris*, Heavy metal, Lead, Siderophore producing bacteria

Industrial and agricultural activities have led to substantial release of toxic metal(s) in the environment, which constitute a major hazard for soil and agriculture ecosystems as well as human health. Contaminant metals can often accumulate in considerable amounts in the plant tissue exceeding the levels that are toxic to man or animal before they produce visible phytotoxic effects. This has caused increasing concern with respect to certain heavy metals, particularly cadmium and lead, which have been implicated as potentially hazardous contaminants in the biosphere [1].

The toxicity of heavy metals such as cadmium, nickel, copper and lead for plant metabolism including photosynthesis is well known [2]. They disrupt the physiological process by binding to protein sulphydryl groups or causing deficiency/ substitution of essential metal(s) in enzymes [3].

It is known that heavy metal pollution causes numerical reduction in soil micro biota, generally followed by the selection and development of heavy metal tolerant microorganisms [4]. The heavy metal enriched environment could lead to the selection of metal tolerant strains in soil capable of plant growth promontory activity. Selection of such heavy metal resistant plant growth promontory rhizobacteria (PGPR) from contaminated sites can contribute to increasing plant biomass by the production of beneficial compounds. Among several mechanisms used for plant growth promotion, siderophore synthesis is well documented due to its iron sequestration from the soil [5-6]. Iron siderophore complex produced by plant growth promontory rhizobacteria are available to be taken up by plant roots and may protect plants from heavy metal induced chlorosis. An appropriate strategy to prevent plant chlorosis due to high level of heavy metal(s) could be to provide them with associated siderophore producing bacteria that supplement a sufficient amount of iron to the plant. In the present study metal resistant bacteria were isolated from contaminated soil and screened on the basis of siderophore production as a growth promoting marker. Maximum siderophore producing bacterial isolate KNP3 was used for *in situ* growth experimentation in metal contaminated soils using *Glycine max* PK042.

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MATERIALS AND METHODS

Selection of lead resistant bacteria

The bacteria were isolated from a soil sample collected from Panki Thermal Power Plant, Kanpur, India. The lead concentration of contaminated soil sample was 1270 µg/kg. Bacteria were isolated on nutrient agar containing lead resistance at 150.00 µM lead acetate concentration. The plates were incubated for two days at 30°C. Lead resistant colonies were subsequently purified on the same media and screened for maximum tolerance level.

Heavy metal analysis

Plant material was processed for metal analysis as described by Burd *et al.* [7] and soil analysis was done by the method described by Gupta [8] using a graphite furnace atomic absorption spectrometer GBC Avanta EGBSFS3000 (Australia).

Data was analyzed by analysis of variance (ANOVA). All ANOVA were significant at 1% level and the mean difference of the treatments was considered to be significant at 5% level.

Siderophore assay

Qualitative estimation was made on chrome azurol S agar (CAS) as described by Schwyn and Neiland [9]. Siderophore quantification was performed as described by Meyer and Abdallah [10]. Briefly, 25ml standard sodium succinate media was inoculated with 100 µl overnight grown culture (OD 0.6) and incubated for 72 h at 120 rpm and 30°C. The culture was centrifuged at 10,000 rpm. Optical Density of supernatant was taken at 400 nm.

16S rDNA sequencing

16S rDNA sequencing was done at the National Center of Cell Science, Pune University Campus, Ganeshkhind, Pune, India. Polymerase chain reaction (PCR) amplification of almost full length 16S rRNA gene was carried out with bacterial primer set 16F27 (5'-CCAGAGTTTGATCMTGGCTCAG-3') and 16R1525XP (5'-TTCTGCAGTCTAGAAGGA GGTGWTCCAGGC-3'). PCR was performed in an automated gene amplification PCR system 9700 thermal cycler (Applied Biosystem, Foster city, USA). The PCR product was sequenced using a Big Dye terminator cycle sequencing kit (V3.1) in an ABI Prism 3730 Genetic Analyzer (Applied Biosystem, USA) to yield a 700-base 5'end sequence which was then analyzed using the RD11 and NCBI database. (Accession no. D.Q.-205432).

In situ characterization

In situ root colonization study was carried out in a polyhouse taking soybean (*Glycine max* PK1042) as test crop. The seeds were coated with bacterial isolate KNP3 using carboxy methylcellulose and were sown in pots using sterile soil (Mollisol/ pH 6.8). To detect the toxicity of lead on soybean, 660 µM lead acetate were added to the soil and pots were kept at 30°C in the polyhouse for 25 days and irrigated with sterile water [7]. Plant height and wet weight were measured before the plant biomass was oven dried. Chlorophyll content of plant leaves was detected according to Hiscox and Israelstam [11].

RESULTS AND DISCUSSION

Isolation of lead resistant bacteria

Maintenance of good soil quality is of prime importance for sustainable agriculture. Soil may become contaminated with heavy metals such as cadmium and lead due to a variety of anthropogenic sources [12]. Even at micro molar concentrations, lead inhibits the growth of most wild types of bacteria and can be tolerated by only a minority of microorganisms. Initial screening of bacteria on lead containing media resulted in the isolation of seventy lead resistant bacteria from polluted soil sample taken from Panki Thermal Power Plant. The screened isolates were found to be tolerant to 250 µM lead concentration. Boularbah *et al*. [13] isolated six cadmium resistant bacteria, which also accumulate heavy metal. *Pseudomonas putida* PhCN contained plasmid that code for cadmium and copper resistance [14].

Lead resistant bacterial isolates were screened for their siderophore production levels. Twenty isolates with a larger zone on CAS agar were chosen for quantitative assay. Isolate showing maximum (126.34 µg/ml) production of siderophore was named KNP3. Isolate KNP3 was tolerant to lead till 1318 µM concentration. At this high heavy metal concentration, the generation time of KNP3 increased to 60±0.86 minutes in lead containing media in comparison to absence of metal (45±1.76 minute).

Table 1 Two-way ANOVA depicting effect of KNP3 on Cd and Pb toxicity of soybean in autoclaved soil under green house at 30°C after 25 days

		Shoot length[b] (cm)	Root length[b] (cm)	Fresh weight[a] (g)	Dry weight[a] (g)	Chlorophyll[c] (mg g^{-1})
Without metal	Mean (Control)	26.0	9.3	1.01	0.31	4.79
	Mean (Treated)	27.9 (7.30)[d]	13.1 (40.0)	1.1 (9.0)	0.34(9.5)	5.08 (6.05)
With Cadmium	Mean (Control)	24.6	6.4	0.85	0.22	2.27
	Mean (Treated)	34.9 (41.86)	10.7 (67.2)	1.46 (71.7)	0.39(77.3)	4.13 (81.9)
Critical difference at 5%		4.76	2.54	0.29	0.29	1.35
With Lead	Mean (Control)	21.10	8.4	0.67	0.17	3.80
	Mean (Treated)	33.80(60.18)	9.5 (13.0)	1.07 (59.7)	0.28(64.7)	4.69 (23.42)
Critical difference at 5%		6.13	4.25[e]	0.26	0.23	1.05

a. Mean of three replicate of 10 plants
b. Mean of ten replicate
c. Mean of four replicate
d. Value in the parentheses [] indicate % increase (↑) over respective control
e. Non-significant

KNP3

GTCTACACATGCAAGTCGAGCGGTAACAGGAGAAAGCTTGCTTTCTTGCTGACGAGCGGCGGACGGGTGAGT
AATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATGACGTCTACG
GACCAAAGCAGGGGCTCTTCGGACCTTGCGCTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGGGGTA
ATGGCTCACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTG
TATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATAAGGTTAATACCCTTATCAA
TTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCG
TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCAGATGTGAAAGCCCCGAGCTTAA
CTTGGGAATTGCATCTGAAACTGGTTGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCACGTGTAGCGGTGA
AATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTAGAGGTTGT
GGTCTTGAACCGTGGCTTCTGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAA
CTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCT
TACCTACTCTTGACATCCAGCGAATCCTTTAGAGATAGAGGAGTGCCTTCGGGAACGCTGAGACAGGTGCTGC
ATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTG
CCAGCGCGTAATGGCGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAA
GTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGAGAAGCGACCTCGCGA
GAGCAAGCGGAACTCATAAAGTCTGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAAT
CGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT
GGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTG
GGGTGAAGTCGT

gi|45922|emb|X07652.1|PVRN16S Proteus vulgaris 16S rRNA gene 2833 0.0 99% similarity
gi|6478168|gb|AF008582.1|AF008582 Proteus mirabilis 16S rib... 2817 0.0 99% similarity
gi|15551728|emb|AJ301683.1|PVU301683 Proteus vulgaris 16S r... 2809 0.0 99% similarity

Fig 1 16s r RNA sequence of *Proteus vulgaris* KNP3 strain

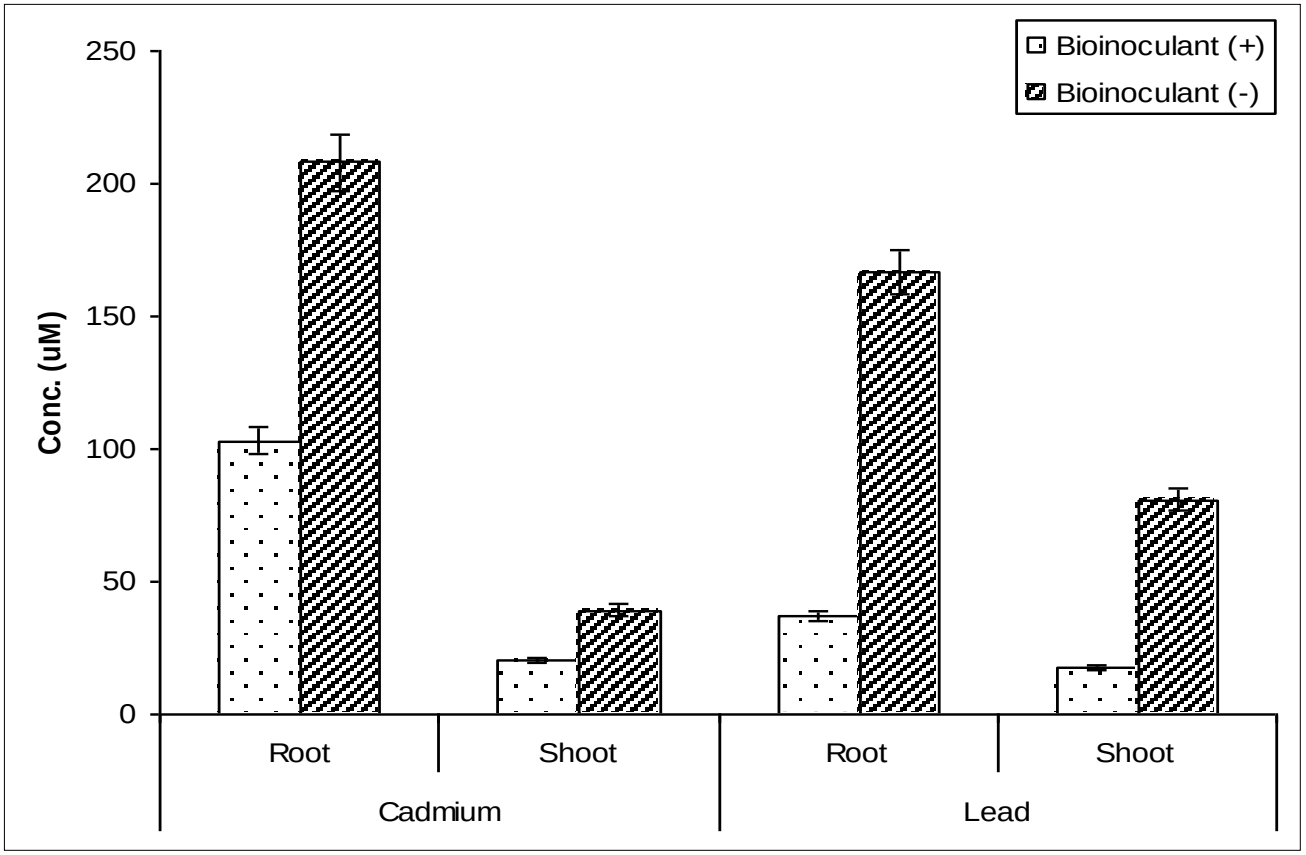

Fig 2 Effect of KNP3 on Cd and Pb accumulation (mean ±SD, n=4) in root and shoot of soybean, in Cd and Pb contaminated soil (where n is the number of replicates)

Uptake of the fluorescent iron siderophore complex provides the organism with a competitive advantage under iron stress. Also, a number of plants are able to use bacterial-iron siderophore complex as a source of iron from the soil [15]. They will also form complexes with metals other than Fe^{3+}, though with a lower affinity. Binding of the siderophore to heavy metal dramatically changes the free metal concentration. Hence it is likely that siderophore production by KNP3 was capable of sequestering heavy metals in addition to iron.

16S rDNA sequencing identified KNP3 as *Proteus vulgaris* (Fig 1). Earlier *Proteus vulgaris* has been reported as a plant growth promoting [16] and heavy metal tolerant strain [17] separately. To the best of our knowledge, this is the first report of a *Proteus vulgaris* strain co-expressing lead tolerance as well as plant growth promoting characteristics.

Bioinoculant application and metal toxicity

To assess the ability *of KNP3* as rhizospheric colonizer in the soil system, a pot experiment was set up with soybean as the plant host. A persistent and statistically significant increase in fresh weight, dry weight and root length (9.0%, 9.0% and 40.0% respectively) was evident in treated plants (Table 1). However, non-significant changes were observed in shoot length of soybean. Moreover, chlorophyll content increased by 6.05% in presence of bioinoculant, which is an indication of the iron utilization of bacterial siderophore complex by soybean plants. Multiple plant growth promontory traits present in individual organism the more likely the chance of that organism being a successful inoculants strain [18].

Excess lead causes a number of toxicity symptoms in plants like stunted growth, chlorosis and blackening of root system. Lead inhibits photosynthesis, upsets mineral nutrition and water balance, changes hormonal status and effects membrane structure and permeability [19]. There was significant decrease in all the agronomical parameters (shoot length, root length, wet weight and dry weight) in lead treated soybean plants (Table 1). KNP3 inoculation significantly increased shoot length, root length, fresh weight and dry weight by 60.18%, 13.0%, 59.7% and 64.7% respectively, in comparison to uninoculated soyabean plant in lead contaminated soil (Table 1). Furthermore, quantity of lead accumulated in bioinoculated plant also decreased to the extent of 78.20% and 69.80% in shoots and roots respectively (Fig 2). Decrease in the lead content absorbed by the plants in presence of *Brevibaciillus* strain. *Microbacterium* exhibited low resistance to cadmium, lead, and arsenate and moderate to arsenite [20]. However, these bacteria reported less percentage of strains with PGP activity, which only included ACC deaminase (23.08%), phosphate solubilization (7.69%), siderophore (30.77%), and IAA production (30.77%) [21].

Treatment with lead caused 20.7% loss of chlorophyll content in soybean. Strong decrease in chlorophyll level of seedlings at the 50 mM of lead treatment [22]. The chlorophyll content in lead treated plants inoculated with KNP3 was found to be equivalent to the chlorophyll content of lead untreated plants (Table 1) Similarly, the ability of *Glomus intraradices* isolate BEG 141 to improve pea growth in cadmium polluted soil [23].

The low iron content of plants due to the presence of heavy metals generally results in chlorosis, since iron deficiency inhibits both chloroplast development and chlorophyll biosynthesis [24]. However, plants can take up microbial iron siderophore complex as an iron source. Therefore, efficient siderophore producing isolates could be ideal bioinoculants for remediation of metal contaminated soil.

Heavy metal toxicity to plants can be reduced by the use of plant growth promoting bacteria. Free-living soil bacteria exert some beneficial effects on plant development when they are either applied to seeds or incorporated in the soil [25]. Metal resistant soil bacterium *Kluyvera ascorbata* promoted the growth of canola (*Brassica compestris*) in the presence of high concentration of nickel, lead and zinc. two strains such as *Pseudomonas fluorescens* (YPS3 GenBank number—MH580200) and *Bacillus safensis* (YKS2 GenBank number—H539636) have s effective chromium degrading capacity up to 84% and 72%, respectively [26]. Similarly, a novel bioremediation system for cadmium based on the symbiosis between leguminous plant and genetically engineered Rhizobia [27]. *Serratia* sp. SY5, which could be applied as a promising microbial inoculant for the direct stimulation of plant biomass production [28]. Similarly, the effects of the inoculation of *Axonopus affinis* with plant growth promoting rhizobacterium and its tolerance to heavy metals as promising bioremediation [29]. Similarly, the effect of *Lactobacillus plantarum* MF042018 has high tolerance against Ni2+ and Cr2+ with potential bioremediation capacity [30]. This work has demonstrated the efficacy of *Proteus vulgaris* KNP3 isolate as a growth promoting bioinoculant for soybean grown in lead contaminated soils.

CONCLUSION

Heavy metals can often accumulate in considerable amounts in the plant tissue and exceed the levels that are toxic to man or animal before they produce visible phytotoxic effects. Plant growth promontory and heavy metal resistant *Proteus vulgaris* KNP3 was able to reduce the metal content from soyabean and moong bean (data not shown) as well as reduce the heavy metal toxicity to the plants. Moreover, beneficial effects on plant development were observed when *Proteus vulgaris* KNP3 was applied as a bioinoculant in heavy metal contaminated soil.

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Conflict of interest

The authors have declared no conflict of interest.

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