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Research Article

ESTIMATION OF PLANT GROWTH PROMOTING POTENTIAL OF TWO NICKEL ACCUMULATING ISOLATES OBTAINED FROM RIVER HOOGHLY ON INDIAN YELLOW MUSTARD (*Brassica hirta*)

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Abstract

Plant growth promoting bacteria (PGPB) are known to influence plant growth by various direct or indirect mechanisms. Present study was conducted with an aim to estimate the PGPB potential of two nickel tolerant bacterial isolates from river Hooghly. Isolates (I-3) (Gram negative coccobacilli) and (II-1) (Gram positive rods) were observed, among a total of 22 other isolates, to tolerate and accumulate significant amounts of nickel and also have multiple Plant Growth Promoting (PGP) activities like IAA production and phosphate solubilization. Present study also shows that seeds of yellow mustard (*Brassica hirta*) inoculated with both the test isolates individually, significantly enhanced root and shoot growth and also protected the plant from the various phytotoxic effects of nickel.

Key words: Nickel accumulation; IAA production; Phosphate solubilization; Plant growth promoting bacteria (PGPB); *Brassica hirta* Bioremediation.

Introduction

Heavy metal pollution of soil is becoming one of the most severe environmental hazards and has received great attention in the last few years because of its negative impact on human health and agriculture. Economically, excessive accumulation of heavy metals is most toxic to crop plants and results in decreased soil microbial activity, soil fertility and yield losses (McGrath *et al.*, 1995). They also threaten human health through the food chain (Biomagnification) (Mclaughlin *et al.*, 1999; Guo *et al.*, 1996; Chen *et al.*, 2003; Sanita *et al.*, 1999). Although Nickel (Ni^{+2}) in general is known as an essential micronutrient and components of some plant enzymes (Eskew *et al.*,1983; Andreeva *et al.*, 2001) but it is highly phytotoxic when present in excessive concentrations. Growth inhibition, changes in chloroplast and chlorophyll concentrations, disturbance of other metabolic and physiological processes especially photosynthesis have been related to Ni^{+2} toxicity (Boominathan and Doran, 2002). Industrialization and various anthropogenic activities have been responsible for increased heavy metal release to the environment. Long term studies on exposure of heavy metals showed decrease in microbial diversity and metabolic processes (Smit *et al.*, 1997). Soil bacteria can be found in the rhizosphere and have been considered to promote plant growth directly or indirectly. Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize plant roots and increase plant growth and yield (Wu *et al.*, 2005; Shrivastava and Kumar, 2011; Shrivastava, 2013). The mechanisms by which PGPRs promote plant growth are thought to include- the ability to produce phytohormones (Egamberdiyeva, 2007), asymbiotic N_2 fixation (Mrkovacki and Milic, 2001), antagonism towards phytopathogenic microorganisms by production of siderophores (and other chelating agents) that can affect trace metal mobility and availability to the plants (Abou-Shanab *et al.*, 2003; Idris *et al.*,.2004) the synthesis of antibiotics, enzymes and/or fungicidal compounds (Ahmad *et al.*, 2006; Jeun *et al*,. 2004) and solubilization of mineral phosphates (and redox changes) and other nutrients (Cattelan *et al.*, 1999).The present study helped us in obtaining more meaningful and realistic knowledge of isolation and characterization of nickel accumulating and plant growth promoting microbes from river Hooghly and their growth promoting and nickel accumulating effects in yellow mustard (*Brassica hirta*)

Materials and methods

Sampling

Fresh river water samples were collected in quasi sterile sampling conditions from three different sites situated in the river Hooghly which are Ratan Babu ghat (Baranagar)(22.64°N 88.37°E), Mahadeo jute mill ghat (Belur)(22°37′57″N 88°21′23″E) and Trunk Terminal near CESC generating centre,(Kashipur)(22.623°N 88.375°E), West Bengal,India. Samples were collected in sterilized glass sampling bottles and transported to the laboratory (at

25±4°C) within 45 minutes of collection for screening of viable and culturable nickel tolerant bacteria. The nickel load of the experimental site fresh river water was measured at SGS enterprise (Kolkata) using standard ICOPES protocol.

Isolation of Nickel tolerant bacteria

The samples were coarsely filtered through sterilized Whatman's No. 1 filter paper to discard solid and large suspended particles. The bacterial isolates were screened by dilution plating of the filtrate (0.2 ml) in Nutrient Agar (NA) media plates (sterile, 90 mm diameter) (g/L): Peptic digest of animal tissue 10, Meat extract 10, Sodium chloride 5, agar 15; final pH 7.2±0.2 at 25°C) containing varied concentrations of Ni^{+2} (mM : 0.5, 1,2,3,4,5,6,7,8,9,10) as analytical grade salts of $NiCl_2$ from their sterilized stocks (100mM). Plates were incubated at 35±2°C for 24 hours. Nickel tolerant bacterial colonies that developed at highest concentration of Ni^{+2} supplemented media were selected as Ni tolerant isolates for further experimental study. The isolates were maintained as axenic cultures by several periodic subculturing onto Ni^{+2} supplemented Nutrient Agar slants.

Assay for phosphate solubilization

Phospahte solubilization assay was performed as per Singh et al (1994) with slight modifications. Pikovaskya agar (g/L: Yeast extract 0.5, Dextrose 10, Calcium phosphate 5, Ammonium sulphate 0.5, Potassium chloride 0.2, Magnesium sulphate 0.1, Manganese sulphate 0.0001, Ferrous sulphate 0.0001, agar 15; pH 7.2±0.2 at 25°C) plates were streak inoculated with a loopful of overnight (18-20 h.) culture (cell density approx. 10^7-10^9 cells/ml assessed turbidometrically at 600 nm) of the bacterial isolates (grown at 35±2°C, 110 rpm in Nutrient broth). Presence of clear zone around the bacterial colonies after 120 hours at 35±2°C indicated positive response to phosphate solubilization.

Assay for detection of Indole acetic acid (IAA) production

IAA quantification was performed following the method of Glickmann *et al*. (1994) with little modifications. Bacterial strains were grown for 72 hours at 35±2°C, 110 rpm on NB with or without sterilized (under 0.21 micron filter) tryptophan (trp) (500 µg /ml) and then the bacterial cells were removed from the culture medium by centrifugation (10,000 rpm, 25°C, and 10 minutes). One ml of the supernatant was mixed vigorously with 4 ml of Salkowski's reagent (150 ml conc. H_2SO_4, 250 ml H_2O, 7.5 ml 0.5 M $FeCl_3$. $6H_2O$), and the absorbance was measured at 535 nm with UV-Visible double beam spectrophotometer (Jasco V-600). The results were done in triplicate and IAA content were measured from standard curve of IAA absorbance performed elsewhere. This assay along with phosphate solubilization was set to be limiting factor screening protocol for further tests to be done.

Heavy metal tolerance assay

Minimum inhibitory concentration (MIC) for the test metal viz. Ni^{+2} were determined using standard tube dilution techniques using Luria broth (g/L; Tryptone 10,yeast extract 5, NaCl 10 ; pH 7.2±0.2 at 25°C) supplemented with different concentrations of Ni^{+2} respectively. Overnight (18-20 hours) bacterial inoculations (parameters as stated before) were used as inoculant. Growth was recorded after 24 hours of incubation at 35±2°C (at 110 rpm for broth cultures). The lowest concentration of metal that completely inhibited microbial growth was considered as the MIC. Development of growth indicated by turbidity in the broth medium was observed and accordingly the MIC was determined.

Cell mediated nickel removal assay

0.25 ml of overnight grown (18-20 h) cell suspensions were inoculated to 75 ml of TMMG (Tris Minimal medium supplemented with glucose [TMMG], (g/L): Tris base: 6.05, glycerol -2- phosphate: 0.67, $(NH_4)_2SO_4$: 0.96, KCl: 0.62, $MgSO_4$: 0.063, $FeSO_4$: 0.0003, glycerol: 0.6; 0.8% glucose; pH was adjusted to 7.00 with 2 M HCl) medium in 250 ml conical flasks having 2 mM of Ni and incubated on a shaking incubator(110 rpm) at 35±2°C. Cells were harvested at 24, 48, 72 and 96 h of incubation by centrifugation (6000 g, 10 min, and 4°C). To analyze the metal content, the medium supernatants were acid digested with nitric acid and perchloric acid (5:3) and the volume was adjusted to a known amount. Nickel content in the supernatants was determined using an atomic absorption spectrophotometer (Perkin Elmer Analyst 400, USA). Sets where cells grew without Ni^{+2} were considered as control. The same protocol was applied to the cell biomass to corroborate the results.

Plant growth promotion and chlorophyll content assay

In situ plant growth promotion studies were done with the two isolates (I-3) and (II-1). Mustard seeds were surface sterilized by incubating with 1:1 (v/v) solution of 30% H_20_2 and sterile distilled water for 30 minutes under aseptic conditions followed by repeated wash with sterile distilled water. The seeds were then imbibed either with sterilized water or with bacterial suspension for one hour and then sown in solarized clay pots (16.5 cm x 10.0 cm x 12 cm) containing sterilized soil. Nickel content of the soil used for plant growth promotion studies was measured at SGS enterprise (Kolkata) using standard USEPA 3052 protocol. Suitable control measures were taken. Seedlings were irrigated every other day either with sterilized water (control) or with 100 ml of 250 µM Ni. The choice of such lowered Ni concentration was chosen to avoid severe effects on test plants. The seedlings were harvested after 10 days and measurement of growth parameters was done. The chlorophyll content was determined from leaf slices weighing one gram by extraction from leaf tissue in 85% acetone following the method of Arnon (1949). Absorbance

of the extract was obtained at 663 nm and 645 nm respectively. Total chlorophyll content (mg /g fresh weight) = [(13×95 × A_{665} – 6×88 × A_{649}) + (24×96 × A_{649} – 7×32 × A_{665}) ×V]/ (1000 × W), with V being the volume of the extract and W being the fresh weight (g) of leaf tissue.

Estimation of Nickel in plants

Microorganisms were removed prior to the estimation of Ni^{+2} in the root samples by vigorous washing with 0.01 M EDTA and sterilized water to avoid interference of Ni^{+2} accumulated by rhizoplane bacteria. The washed root samples or shoot samples were then dried at 105°C and were digested in a mixture of conc. HNO_3 and $HClO_4$ (4:1, v/v) (Chen *et al*., 2003). The Ni^{+2} content in the digest was determined by atomic absorption spectrometer (AAS).

Statistical analysis

Analysis of variance (ANOVA) followed by Tukey's HSD Test ($p < 0.05$) were used to compare treatment means.

Result and Discussions

Screening for Nickel resistant isolates

Heavy metal resistant bacterial colonies from fresh river water, containing 1.83 ppm residual nickel concentration, were found in all the samples ranging up to 10 mM concentration of Ni^{+2} in solid slant assay. Initially 22 different colony isolates had been identified as given in Fig. 1.

Assay for phosphate solubilization

The two isolates were chosen on the basis of high level of phosphate solubilizing activity, as in Fig. 2. One is third isolate of sample I (I-3) and another is the first isolate of sample II (II-1). These two isolates were selected for further assays.

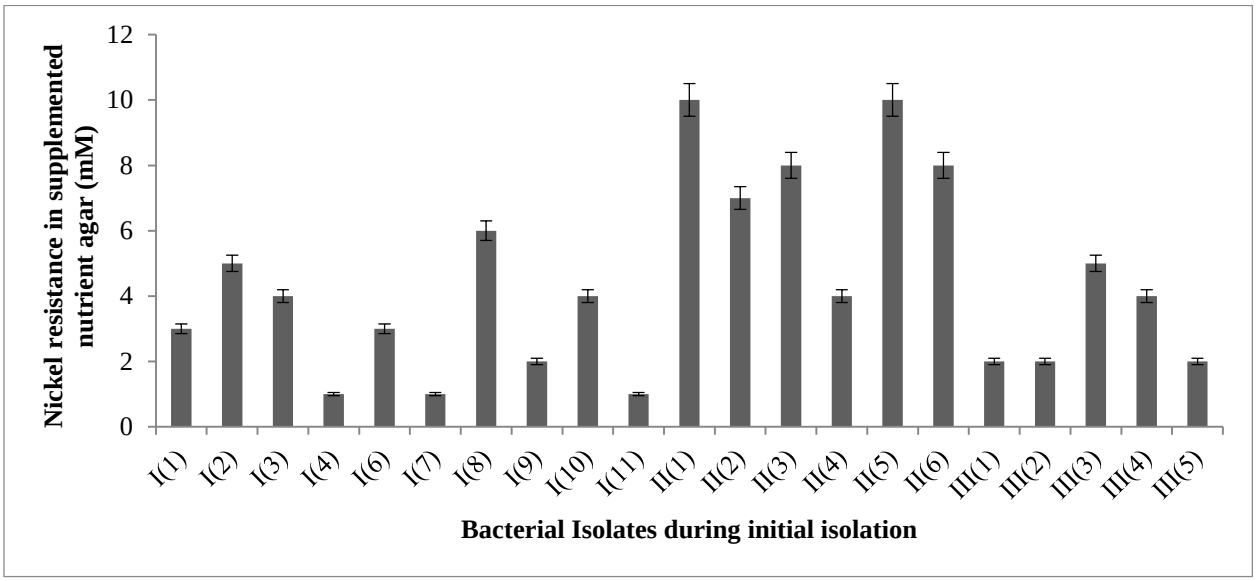
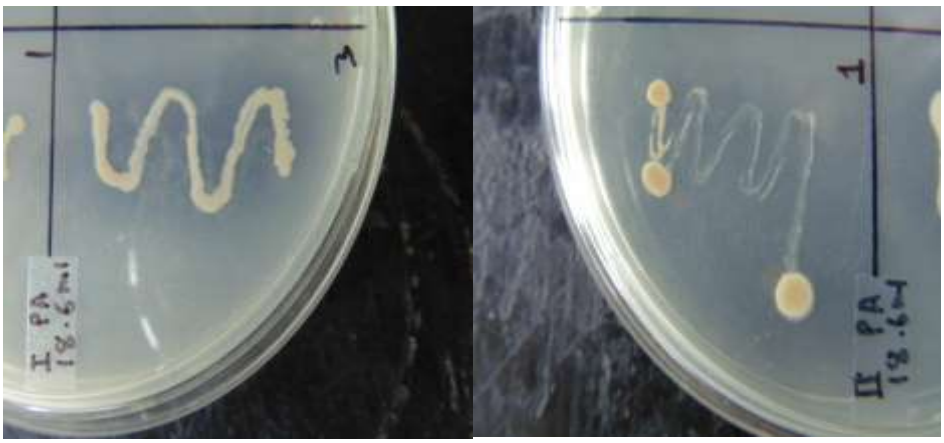


Fig. 1: Comparative Nickel tolerance potential of all the different isolates isolated from the 3 sampling habitats (I, II, III). The result is a mean of 5 assays done for each particular isolate.



A B

Fig. 2: Initial screening profile of the two isolates in Pikovskaya agar, A: isolate I (3) and B: isolate II (1)

Indole acetic acid (IAA) production assay

Selected isolates gave positive results for IAA production and the amount of production was significant for both isolates (I-3 and II-1) as given in Fig. 3

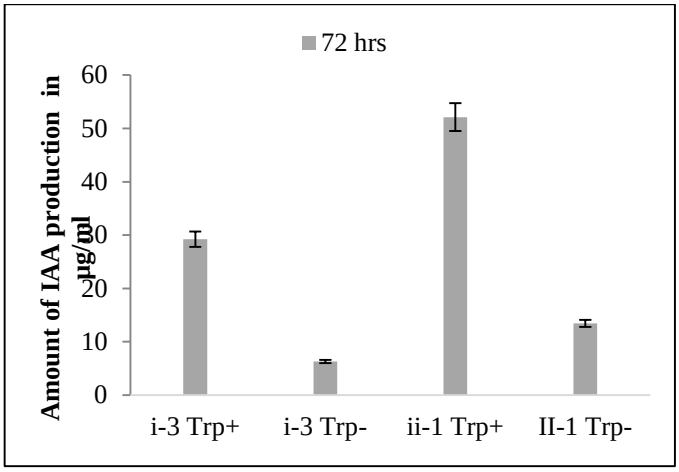


Fig:-3 comparison between the amount of IAA production between the two selected isolate i.e. (I-3) & (II-1). The experiment is done in triplet and the graph shows mean profile with ±1 unit error.

Heavy metal tolerance assay

MIC of both the isolates in LB medium were determined for Ni^{2+}.Selected isolates shows high level of nickel tolerance of which (I-3) have MIC value of 2mM in LB while (II-1) shows a significant 8 mM value of MIC in LB as shown in Table 1.

Nickel removal assay

Fig. 4 indicate, the percentage of nickel removed from the medium supernatant with time, study indicates that with time the amount of nickel removed by the two isolates increased significantly. Fig. 5 indicate the amount of nickel present in the bacterial pellet in corroboration to depletion from supernatant. With time the nickel content in the bacterial pellet increased significantly. The result shows that both the isolates were nickel accumulators and the study revealed that isolates (II-1) and (I-3) accumulates 56.94% and 31.12% respectively after a period of 96 hours. The results show that there was no nickel quenching by medium particles and all the nickel were accumulated by the two isolate.

Plant growth promotion and chlorophyll content assay

As shown in Fig. 6 and Fig. 7 the comparative root and shoot length of pot experiments for the two selected isolates without and with nickel stress respectively in sterilized soil containing residual nickel load of 23.67 mg/kg. Study indicates a significant difference between the heights of yellow mustard plants (Brassica hirta) between the inoculated and negative control pots visually shown in Fig:-8. Seedlings from inoculated seeds have significantly greater root [I(3) by 77.3% increase and II(1) by 67.2% increase] and shoot[I(3) by 24.2% increase and II(1) and 27.7% increase] compared to control, both in presence and absence of nickel stresses, in presence of nickel stress shoot length of isolates I(3) and II(1) were increased by 23.2% and 20.1% and for the same root length was also found to increase by 38.8% and 40.7% . Heights of control plants and the plants from nickel containing pots showed a nickel cause decrease of root and shoot by 30.3% and 5.55% respectively and the phytotoxic effects like spots in leaves, yellowing, curling and blackening of leaves are less prominent in plants from nickel and inoculated seed containing pots shown in Fig:-9. (Table 2) Shows significant chlorophyll loss in plants of nickel containing negative control pots but not significantly affected chlorophyll content level in plants taken from nickel and inoculated seed containing pots. These results built a presumptive idea of both the plant growth promoting and nickel accumulating potential for both the isolates.

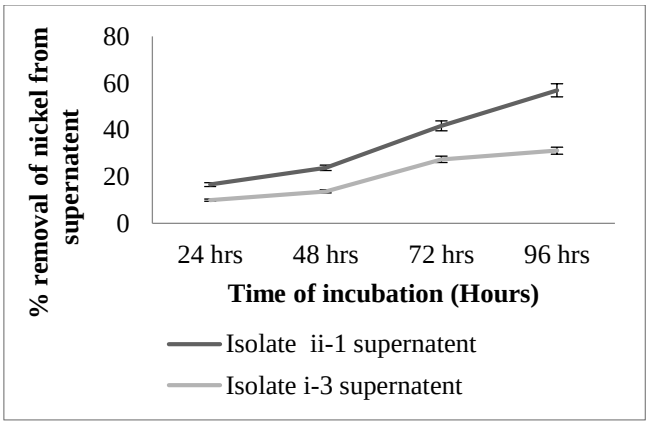


Fig. 4: Percentage removal of nickel from supernatant, determined using an atomic absorption spectrophotometer (Perkin Elmer Analyst 400, USA). The result is a mean of 5 assays and the graph shows mean profile with ±1 unit error.

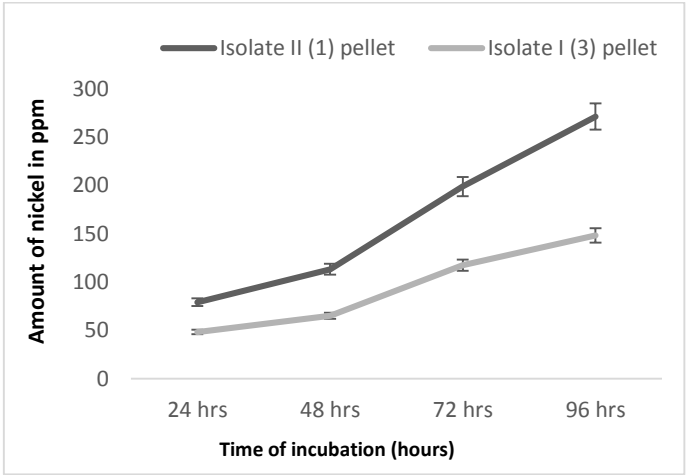


Fig. 5: Amount of nickel content in the bacterial pellet, determined using an atomic absorption spectrophotometer (Perkin Elmer Analyst 400, USA). The result is a mean of 5 assays and the graph shows mean profile with ±1 unit error.

Table 1: Comparative Ni^{+2} MIC assay between the two isolates

	Ni+2 conc. (mM) in Luria broth												
Isolates	**Inoculum Blank**	**0.5**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
I(3)	-	+	+	+	-	-	-	-	-	-	-	-	-
II(1)	-	+	+	+	+	+	+	+	+	+	-	-	-

("+" denotes growth, "-"denotes no growth)

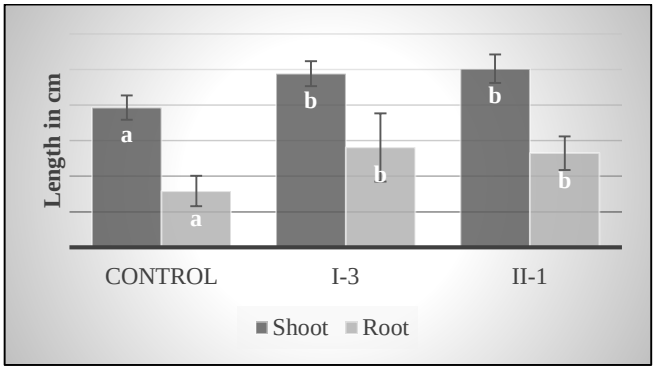


Fig. 6: Plant growth promotion by two isolates without nickel stress: Shoot and root elongation of *Brassica hirta*. Results are expressed as mean. One way ANOVA was performed for each group. Means (±SD) for the same plant section with different letters are significantly different from each other ($P < 0.05$) according to the Tukey's HSD Test. The F-values of ANOVA for shoot and root lengths are $F = 20.6$ ($P < 0.0001$) and $F = 9.08$ ($P < 0.01$), respectively

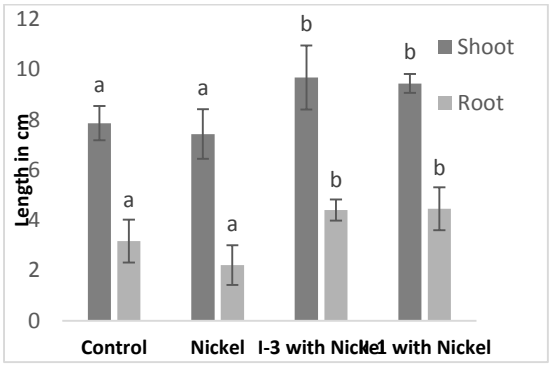
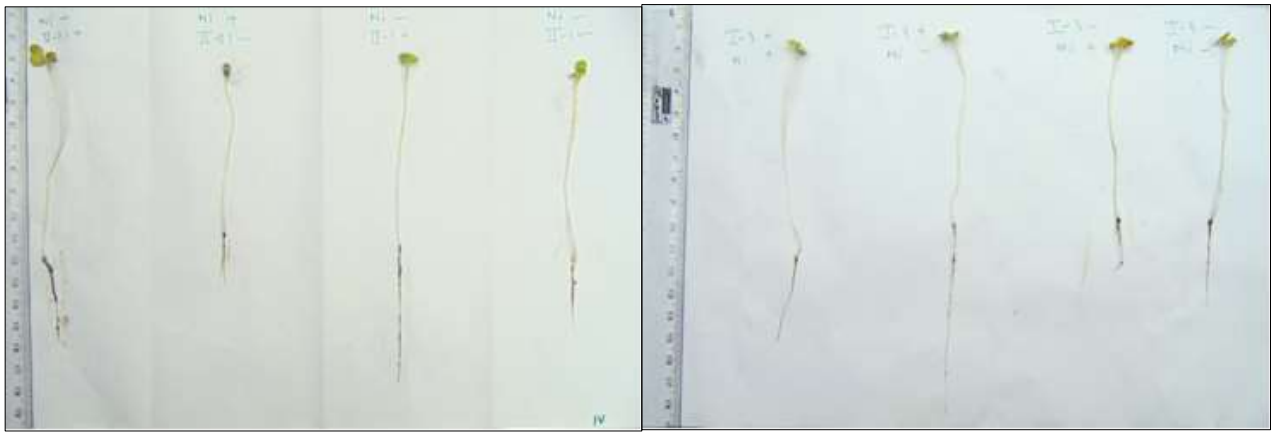


Fig. 7: Plant growth promotion by two isolates with nickel stress**:** Shoot and root elongation of *Brassica hirta.* Results are expressed as mean. One way ANOVA was performed for each group. Means (±SD) for the same plant section with different letters are significantly different from each other ($P < 0.05$) according to the Tukey's HSD Test. The F-values of ANOVA for shoot and root lengths are $F = 11.05$ ($P < 0.0001$) and $F = 13.6$ ($P<0.0001$), respectively.



A B

Fig. 8: Comparative visual growth profile of root and shoot lengths in of yellow mustard plant used in pot experiment of (I-3) (A) and (II-1) (B). In pots with nickel contamination, nickel water is given at 250 µM strength in 100 ml distilled water and in nickel (-) pots only 100ml of distilled water is given. Water is given in every day. Plants were harvested after 10 days.

Table 2: Comparative chlorophyll content study between the two isolates,

Chlorophyll content (mg g^{-1})	Pot experiment with II(1) and I(3) as inoculum					
	Nickel and inoculum blank	**Nickel blank + inoculum**	**Nickel + inoculum blank**	**Nickel + inoculum**	**MS**	**CD (at 5%)**
I(3) as inoculum	2.310 (±0.074)	2.650 (±0.021)	1.520 (±0.024)	2.140 (± 0.052)	0.024	0.143
II(1) as inoculum	2.280 (±0.089)	2.770 (±0.027)	1.480 (±0.036)	2.220 (± 0.033)	0.027	0.147

(Data for each treatment regime are the mean of 10 observations with SD in parenthesis; CD (*P <0.05) extracted from ANOVA)

Fig. 9: (A, B, C and D) Phytotoxic effect of Nickel in yellow mustard plant, in pots which are contaminated with nickel but are lacking bacterial inoculation. A and B show loss of leaf surface area and chlorophyll as spots appear. C and D show plant necrosis and stunted growth.

Effect of the isolates in reducing Ni^{+2} load in plants

The colonization of the rhizospheric environment of the plants by both the isolates reduced Ni^{+2} load significantly on the different plant parts as in Fig:-13 leading to a decrease of 40.85% in root and 16.17% in shoot accumulation of Ni^{+2} in case of I (3) whereas a decrease of 63.14% in root and 39.38% in shoot accumulation in case of II (1) assisted bioremediation.

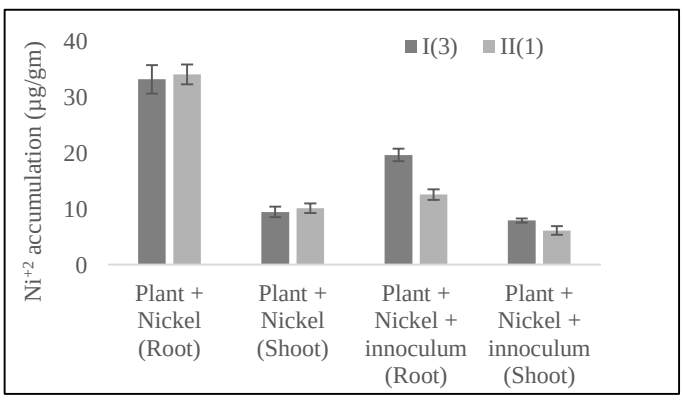


Fig. 10: Comparative effect of the two isolates on Ni^{+2} accumulations (±1 S.D.) in different morphology of yellow mustard (*Brassica hirta*)

Conclusion

The present study revealed that the two isolates (I-3) and (II-1) have plant growth promoting and heavy metal accumulating activity. Further insights into their molecular mechanisms would be done in the future eventually leading to the platform from where it can be decided whether it can be used as biofertilizer for promoting plant growth (especially crop plants) and can also be used as a bioremidiator in Nickel affected areas.

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Reference

Abou-Shanab RA, Angle JS, Delorme TA, Chaney RL, Berkum P van, Moawad H, Ghanem K and Ghozlan HA (2003)

Rhizobacterial effects on nickel extraction from soil and uptake by Alyssum murale. *New Phytol.* **158**: 219–224 DOI: 10.1046/j.1469-8137.2003.00721.x

Ahmad F, Ahmad I and Khan MS (2006) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbial. Res.* **36**: 1-9 DOI: 10.1016/j.micres.2006.04.001

Andreeva IV, Govorina VV, Vinogradova SB, and Yagodin BA (2001) Nickel in Plants. *Agrokhimiya*. **3**: 82–94.

Arnon DI (1949) Copper enzymes in isolated chloroplast. Polyphenol oxidase in Beta vulgaris. *Plant Physiol*. **24** 1-15. DOI:10.1104/pp.24.1.1

Boominathan R and Doran P (2002) Nickel-induced oxidative stress in roots of the hyper accumulator *Alyssum bertolonii. New Phytol*. **156**: 205-215.DOI:10.1046/j.1469-8137.2002.00506.x

Cattelan AJ, Hartel PG and Fuhrmann JJ (1999) Screening for plant growth-promoting rhizobacteria to promote early soybean growth. *Soil Sci. Soc. Am. J.* **63**: 1670-1680. DOI:10.2136/sssaj1999.6361670x

Chen Y, Yang Y, Zheng S and Wong M (2003) Effect of cadmium on nodulation and N_2 fixation of soyabean in contaminated soils. *Chemosphere* **50**: 781-787.

Egamberdiyeva D (2007) The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. *Appl. Soil. Eco*. **36**: 184-189 DOI:10.1016/j.apsoil.2007.02.005

Eskew DL, Welch RM, and Cary EE (1983) Nickel: An Essential Micronutrient for Legumes and Possibly All Higher Plants. *Science* **222**: 621–623. DOI: 10.1126/science.222.4624.621

Glickmann E and Dessaux Y (1994) A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. *Appl. Environ. Microbiol.* **61**: 793–796.

Guo Y, George E and Marschner H (1996) Contribution of an arbuscularmycorrhizal fungus to the uptake of cadmium and nickel in bean and maize plants. *Plant and soil* **184**: 195-205. DOI: 10.1007/BF00010449

Idris R, Trifonova R, Puschenreiter M, Wenzel W, Sessitsch A (2004), Bacterial communities associated with flowering plants of the Ni hyperaccumulator Thaspigoesingense. *Appl. Environ. Microbiol.* **70**: 2667–2677. DOI: 10.1128/AEM.70.5.2667-2677.2004

Jeun YC, Park KS, Kim CH, Fowler WD and Kloepper JW (2004) Cytological observations of cucumber plants during induced resistance elicited by rhizobacteria. *Biol. Control* **29**: 34–42. DOI: 10.1016/S1049-9644(03)00082-3

McGrath SP, Chaudri AM and Giller KE (1995) Long-term effects of metals in sewage sludge on soils, microorganisms and plants. *J. Ind. Microbiol.* **14**(2): 94–104. DOI: 10.1007/BF01569890

Mclaughlin MJ, Parker DR, Clarke JM (1999) Metals and micronutrients-food safety issues. *Field Crops Res.* **60**: 143–163. DOI:10.1016/S0378-4290(98)00137-3

Mrkovacki N, and Milic V (2001) Use of Azotobacter chroococcum as potentially useful in agricultural application. *Ann. Microbiol.* **51**: 145– 158.

Sanita di Toppi L and Gabbrielli R (1999) Response to cadmium in higher plants. *Environ. Exp. Bot.* **41**: 105-130. DOI: 10.1016/S0098-8472(98)00058-6.

Singh S and Kapoor KK (1994) Solubilization of insoluble phosphates by bacteria isolated from different sources. *Environ. Ecol.* **12**: 51–55.

Smit E, Leeflag P and Wernars, K (1997) Detection of shifts in microbial community structure and diversity in soil caused by copper contamination using amplified ribosomal DNA restriction analysis. *FEMS Microbiol. Ecol.* **23**: 249-261. DOI: 10.1111/j.1574-6941.1997.tb00407.x

Shrivastava UP and Kumar A (2011) Biochemical Characterization of Siderophore Producing Plant Growth Promoting Rhizobacteria (PGPR) Isolated from Rice Rhizosphere. *Nep. J. Integrated Sci.* **1**: 31-37.

Shrivastava UP (2013) Isolation and initial characterization of diazotrophic plant growth promoting rhizobacteria (PGPR) from rice rhizosphere of Parsa and Bara district of Nepal. *Int. J. Pharma. Life Sci.* **4**: 2481-2488.

Wu SC, Cao ZH, Li ZG, Cheung KC and Wong MH, (2005) Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. *Geoderma*. **125**: 155–166. DOI: 10.1016/j.geoderma.2004.07.003