



Plant growth promotion by *Bradyrhizobium japonicum* under heavy metal stress



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ABSTRACT

The increase in usage of heavy metals in different industrial activities causes their existence in effluents. Excessive concentrations of these heavy metals pollute soil and water. Heavy metals cause toxicities and other harmful effects not only in humans and animals but also in plants and soil microorganisms. Heavy metals disrupt many biochemical and physiological activities in bacteria, including growth, development, enzyme and hormone production. Indole acetic acid (IAA) is one of the most important hormones in plants, which is secreted by both bacteria and plants. The present study assessed the effects of Ni, Pb and Cu on the growth of *Bradyrhizobium japonicum* and IAA production.

B. japonicum were grown in yeast mannitol broth in a rotary shaker at 100 rpm for 18 h for each variant. Ni, Pb and Cu with concentrations ranging from 2.5–40 mg/L were added to *B. japonicum* cultures and turbidity was measured at 600 nm. Effects of Ni, Pb and Cu on IAA production were determined by measuring the IAA content with Salkowski method. The metal uptake by *B. japonicum* was determined in the range of 5–50 mg/L. A lettuce seedling assay was conducted to observe the effect of *B. japonicum* on plant growth under heavy metal stress. Lettuce seedlings inoculated with the bacterium were spiked with 5–500 mg/L of Ni, Pb and Cu. Shoot and root length, and plant dry biomass were measured after 5 days of germination.

Bacterial growth was reduced with the increase of Ni and Cu concentrations. However, there was no significant growth effect with Pb in the considered range. The IAA production also followed the same pattern with Pb and Ni, but in the presence of Cu, IAA content was slightly increased and reached an equilibrium. The growth retardations observed in lettuce in the presence of Pb, Ni and Cu were in the decreasing order of Ni > Cu > Pb. Bacterial inoculation reduced the heavy metal stress and increased the shoot and root lengths of lettuce seedlings. FTIR spectrum of the bacterial biomass showed that amine and nitro groups are responsible in metal sorption process. The Pb-treated bacterial biomass showed a considerable difference in the FTIR spectrum compared to other treatments. According to our results, *B. japonicum* is sensitive to heavy metal stress with a retardation of growth and IAA production, though it is able to enhance the growth of lettuce seedlings under heavy metal stress.

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1. Introduction

The amount of hazardous material released into the environment has increased with the rise of technology and industrial agriculture (Neilson and Rajakaruna, 2015). Industrial waste, landfill leachate, sewage sludge, excess fertilizer application, insecticides and fungicides release pollutants into soil and water (Ray et al., 1986; Gimeno-García et al., 1996; Järup, 2003). Organic and inorganic pollutants, including heavy metals are the major categories of pollutants (Boyd and

Rajakaruna, 2013). Unlike other pollutants, heavy metals are hazardous, since they cannot be degraded by biological or chemical means (Montazer-Rahmati et al., 2011), leading to harmful effects on ecosystems, including bioaccumulation (Lugauskas et al., 2005).

Lead (Pb) is considered as one of the most common and toxic environmental contaminants found in soils (Gall and Rajakaruna, 2013). Unlike other metals, Pb has no biological role, and is potentially toxic to all organisms, including microbes (Sobolev and Begonia, 2008). Another frequently found heavy metal in soil is copper (Cu), which is an active ingredient of fungicides due to its toxicity on pathogens, including soil microbes (Dussault et al., 2008). Nickel (Ni) is also a commonly found heavy metal in soils, especially in soils associated with serpentinite

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and other ultramafic bedrocks (Vithanage et al., 2014). Salts enriched with Ni are effective systemic fungicides and therefore used for controlling cereal rusts (Mishra and Kar, 1974). Nickel can cause permanent soil contamination due to its specific physicochemical properties and mechanisms of biochemical action (Aydinalpi and Marinova, 2003).

The presence of high concentrations of heavy metals can alter the diversity and structure of soil microbial populations, including their reproduction and other physiological processes (Arnebrant et al., 1987; Giller et al., 2009). Plant–microbial interactions in the rhizosphere are important determinants of plant health and soil fertility (Babalola, 2010; Neilson and Rajakaruna, 2012; Pérez-Montañó et al., 2014). The presence of plant growth promoting bacteria may prevent deleterious effects of phytopathogenic organisms by the production of siderophores, (Rungin et al., 2012) HCN (Keel and Défago, 1997), antibiotics (Klopper et al., 1989), and fungal cell wall-degrading enzymes, including chitinases and β -1,3-glucanases (Inbar and Chet, 1991). Additionally, they may directly affect plant growth by promoting the production of plant hormones such as auxins, cytokinins, gibberellins, ethylene and abscisic acid (Barea et al., 1976; Glick, 1995; Hayat et al., 2010).

Indole acetic acid is one of the most important auxin-type plant hormones (Mano and Nemoto, 2012). It is secreted by both soil microbes and plant roots. The combination of both bacterial and endogenous plant IAA stimulates plant cell proliferation (Glick et al., 1995; Glick et al., 2007). IAA is synthesized by plant-associated microbes via tryptophan (trp)-dependent and -independent pathways. The trp-dependent pathways are indole-3-acetamide (IAM), indole-3-pyruvate (IPyA), and trp side-chain oxidase pathways (Costacurta and Vanderleyden, 1995). The presence of high heavy metal concentrations in soil may cause detrimental effects on both soil microbes and plants (Aydinalpi and Marinova, 2003). Heavy metals act as inhibitors of many biochemical processes (Arnebrant et al., 1987; Aydinalpi and Marinova, 2003), such as enzyme and hormone production. Therefore, the presence of heavy metals in high concentrations may reduce the production of IAA and thereby reduce the growth of microorganisms (Arnebrant et al., 1987; Aydinalpi and Marinova, 2003). This study was conducted to 1) assess the growth and IAA production of *Bradyrhizobium japonicum* in the presence of Pb, Cu and Ni and 2) to determine the effect of *B. japonicum* inoculation on lettuce seedlings under heavy metal stress.

2. Materials and methods

2.1. *B. japonicum* growth under heavy metal stress

Pb, Cu and Ni concentrations of 2.5, 5, 10, 20, 30, 40 mg/L were incorporated into 20 ml each of yeast mannitol broth (YMB) in 100 ml Erlenmeyer flasks. Each sample was inoculated with an overnight incubated *B. japonicum* culture with an optical density of 0.5 at 600 nm. After inoculation, the samples were incubated at 30 °C in a rotary shaker at 100 rpm for 18 h. The optical density was measured at 600 nm using a UV spectrophotometer (Shimadzu UV-2450).

2.2. Heavy metal uptake by *B. japonicum*

Pb, Cu and Ni were each incorporated into 20 ml of sterile distilled water inoculated with 0.5 g of bacterial culture in 100 ml Erlenmeyer flasks in concentrations, 5, 50, 250, 500 mg/L. They were equilibrated for 10 h in a rotary shaker at 100 rpm at 30 °C. The samples were centrifuged at 6000 rpm for 20 min and the supernatant was subjected to Atomic Absorption Spectrophotometry (GBC AA933) to determine the equilibrium concentrations of Pb, Cu and Ni.

2.3. FTIR investigations

The heavy metal equilibrated bacterial pellets obtained after centrifugation were air dried to remove excess water. Pellets were prepared by mixing 0.01 mg of dried sample with 100 mg of KBr. The pellets

were subjected to FTIR (Fourier Transformed Infrared Spectroscopy) to determine the surface adsorption of metal ions.

2.4. Root and shoot elongation assay

Lettuce seeds (*Lactuca sativa*) were used for the seedling assay. Seeds were surface sterilized by soaking for 10 min in 1.5% sodium hypochlorite and then thoroughly rinsed with sterile distilled water. The sterilized seeds were incubated for 1 h at room temperature in sterile distilled water. Fifteen overnight-germinated lettuce seedlings were placed in each Petri dish lined with a cellulose filter paper. A bacterial suspension with optical density 0.573 at 600 nm was used for seed inoculation. The seeds were incubated at 25 °C for 5 days under 16/08 h light and dark conditions. One milliliter of Pb, Cu and Ni each in concentrations 5, 50, 250, 500 mg/L was added separately to each seed sample with and without the bacterial inoculation. Seeds soaked in sterile distilled water were used as control. Each treatment was replicated twice. At the end of 5 days of growth, the seedling shoot and root length and plant dry weight were determined.

2.5. Effects of heavy metals on IAA production by *B. japonicum*

Yeast mannitol broth with 0.1% tryptophan was spiked with Pb, Cu and Ni in a range of 2.5–40 mg/L. One milliliter of bacterial suspension with optical density 0.573 at 600 nm was used to inoculate each sample in 100 ml Erlenmeyer flasks. Samples were incubated at 30 °C in a rotary shaker at 100 rpm for 18 h. The broth was centrifuged after incubation. Supernatant was reserved and 1 ml was mixed with 2 ml of Salkowski's reagent (2% 0.5 FeCl₃ in 35% HClO₄ solution) and kept in the dark. The optical density (OD) was measured at 530 nm after 30 min (Ehmann, 1977; Fuentes-Ramirez et al., 1993).

2.6. Data analysis

Data were analyzed by Analysis of Variance (ANOVA) in SAS (9.1) statistical package. Means were compared using Duncan's Multiple Range Test (DNMRT) at $p < 0.05$.

3. Results

3.1. Effects of heavy metal concentration on bacterial growth

Turbidity of the *B. japonicum* culture incubated for 18 h with Pb, Cu and Ni is shown in Fig. 1. It shows a rapid decrease in turbidity up to 5 mg/L of Pb, then a gradual increase, with the maximum turbidity at 30 mg/L of Pb. In the case of Cu, a gradual decrease in turbidity was observed as the Cu concentration was increased, and there was a slight increment of turbidity observed beyond 20 mg/L. Ni shows a gradual decrease in turbidity as the Ni concentration is increased, reaching an equilibrium after 10 mg/L.

3.2. Effects of heavy metals on IAA production by *B. japonicum*

Fig. 2 shows the IAA production by *B. japonicum* under the influence of Pb, Cu and Ni stress. Cu leads to a reduction in IAA production at low concentrations, however, as the metal concentration is increased, the IAA production was also increased, with further increase of the metal causing a gradual reduction of IAA. IAA production pattern under Ni stress was similar to that shown for turbidity under Ni stress. Pb leads to a small increment of IAA production at low concentrations but a considerable change in IAA production is not seen as the Pb concentration is increased.

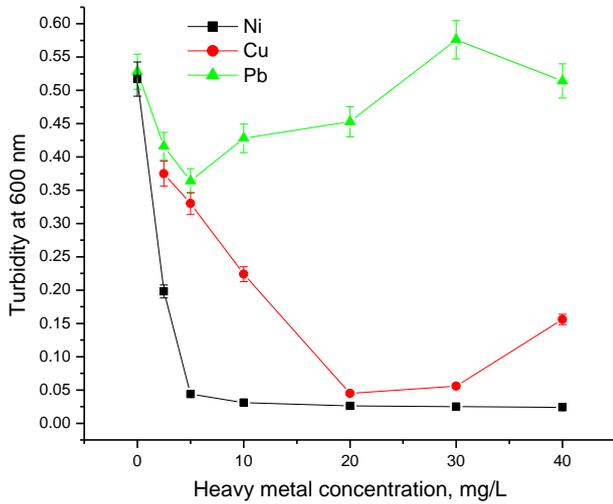


Fig. 1. Effects of Pb, Cu and Ni on *B. japonicum* cells incubated at 30 °C for 18 h. Different letters indicate significant differences ($p = 0.05$, $df = 2$). Error bars represent the standard error of the mean.

3.3. Heavy metal uptake by *B. japonicum*

Pb, Ni and Cu uptake by *B. japonicum* is shown in Fig. 3. As the metal concentration is increased metal uptake was also increased. In the case of Ni, the metal removal by the bacterium was increased as the initial metal concentration increased, reaching an equilibrium at 30 mg/L. However, both Pb and Cu did not come to an equilibrium within the experimental range.

Plant assay shoot and root lengths of lettuce seedlings are shown in Fig. 4 and Fig. 5, respectively. As the Ni and Pb concentrations increase, the shoot length was decreased, whereas in the case of Cu, the increase in concentration did not affect the shoot length until 250 mg/L. However, shoot length showed a 74% reduction at 500 ppm of Cu. Overall, the highest shoot inhibition was observed with Ni. In the case of root growth, all three metals (Ni, Cu and Pb) showed an inhibitory effect. As the metal concentration was increased, roots showed a gradual reduction in length under Ni, Cu and Pb. However, seedlings showed an increment in shoot length under Cu when inoculated with bacteria, but the increment was significant only at 5 mg/L of Cu, which showed the highest shoot length increment. Shoot length also showed a significant increment when inoculated with bacteria at 5 mg/L of Ni.

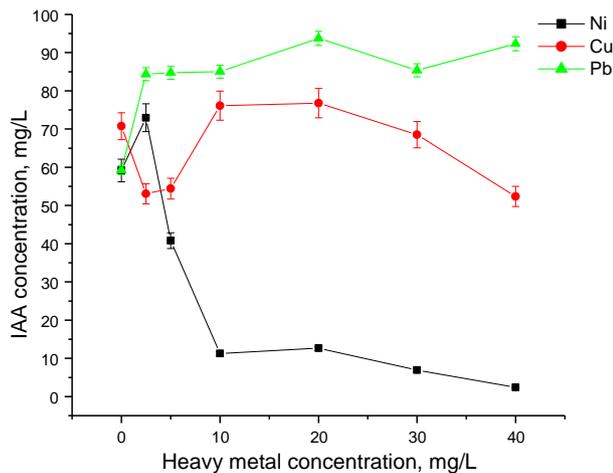


Fig. 2. IAA production by *B. japonicum* under Pb, Cu and Ni stress. Cells were grown in YMB supplemented with 0.1% tryptophan. Different letters indicate significant differences ($p = 0.05$, $df = 2$). Error bars represent the standard error of the mean.

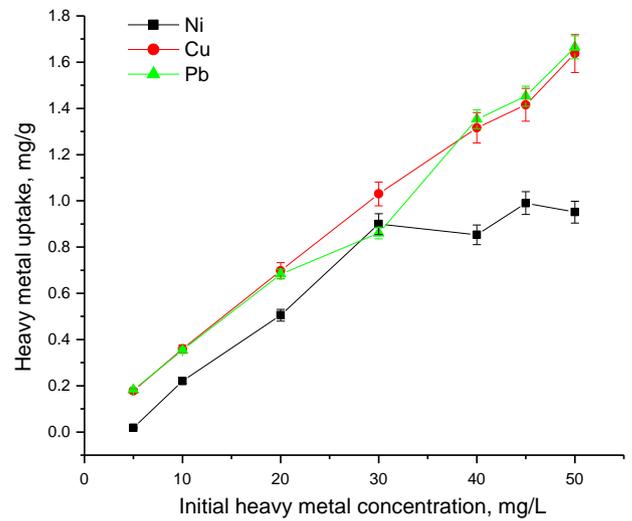


Fig. 3. Pb, Cu and Ni uptake by *B. japonicum* with an initial metal concentration range from 5–50 mg/L. Different letters indicate significant differences ($p = 0.05$, $df = 2$). Error bars represent the standard error of the mean.

In the presence of 50 ppm of Ni, the bacteria were not able to have a beneficial effect on shoot length. Even in the presence of bacteria, seedlings died under 250 and 500 mg/L Ni concentrations. Interestingly, in the presence of bacteria, all Pb concentrations led to a significant increase in shoot length. In the case of roots, the lengths were decreased as Cu, Ni and Pb concentrations increased. At 5 mg/L of Cu, Ni and Pb, the root lengths were significantly increased under bacterial inoculation. However, at 50, 250, 500 mg/L of Cu, there were no increases in root lengths. Although not significant, there was an increase in root length at 50 mg/L Ni. Under all Pb concentrations, the bacteria had a positive effect on the root length of lettuce seedlings. This indicates the beneficial effect of bacteria, which is able to reduce the negative growth effects caused by heavy metals (See Figs. 4, 5).

3.4. FTIR investigations

FTIR spectrum of broad and strong bands were observed in the region of 3200–3600 cm^{-1} as shown in Fig. 6. Few medium peaks were observed at 1,080–1360 cm^{-1} , and 1550–1640 cm^{-1} , respectively. Two strong bands were seen in 1515–1560 cm^{-1} and 1345–1385 cm^{-1} . Few changes were observed in the FTIR of the heavy metal loaded bacteria. Narrowing and increasing of height in the peak at 1000–1100 cm^{-1} were seen in Cu-loaded bacterial biomass whereas peak sharpening and shifting were observed at 1200–1300 to 1230 cm^{-1} . Ni-treated biomass exhibited new peaks in the region of 1080–1360 cm^{-1} . Peak disappearances were observed in the 1200–1300 cm^{-1} region and at 1450 cm^{-1} in the FTIR of the Pb-loaded bacteria.

4. Discussion

Heavy metals cause toxic physiological and functional impairments in both microorganisms and plants (Araújo and Monteiro, 2005; Atici et al., 2005). Our study examined the effects of Pb, Ni and Cu on the growth of *B. japonicum* and lettuce seedlings in the presence or absence of the bacterium. Pb is not known to be of any biological importance and is toxic to cellular activities even at very low concentrations (Bruins et al., 2000). It negatively influences the growth and metabolism of microbes (Levy et al., 1999; Geebelen et al., 2002; Jarosławiecka and Piotrowska-Seget, 2014). Even though it is toxic, many microorganisms have evolved stress tolerance mechanisms, enabling them to survive under Pb exposure (Jarosławiecka and Piotrowska-Seget, 2014). Both Cu and Ni act as micronutrients for plants under low concentrations (Epstein, 1972; Dalton et al., 1988; Mortvedt, 1991; Fu and Maier,

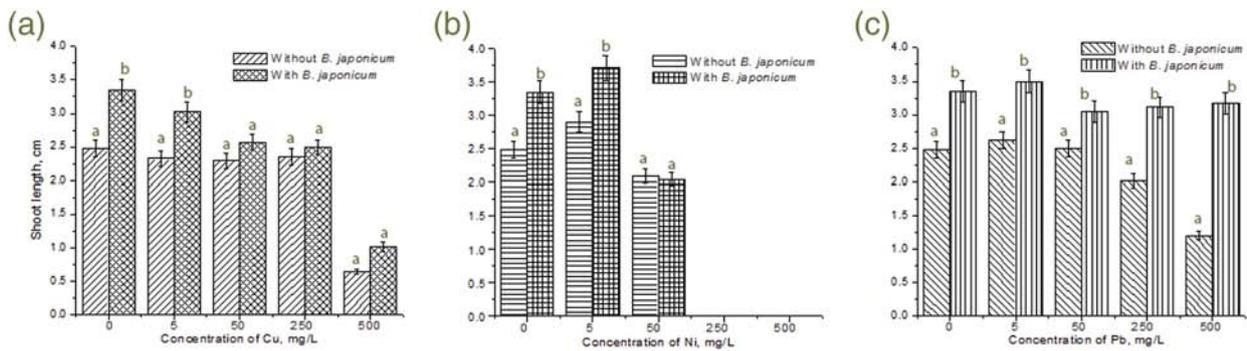


Fig. 4. Effect of *B. japonicum* on shoot growth of *Lactuca sativa* seedlings under Pb, Cu and Ni. Different letters indicate significant differences ($p = 0.05$, $df = 8$). Error bars represent the standard error of the mean.

1992). Ni is an essential component of urease in living cells (Polacco et al., 2013). A deficiency of Ni in plants results in necrotic lesions in leaves in response to toxic accumulations of urea (Dalton et al., 1988) and reduced flowering in a Ni-hyperaccumulating plant endemic to serpentine soil (Ghasemi et al., 2014). Cu acts as an essential component of many biochemical reactions in living cells (Kabata-Pendias, 2010). Both these trace nutrient elements are toxic to plants and microbes under high concentrations (Trevers and Cotter, 1990; Kabata-Pendias, 2010).

Our study documents differential and concentration-dependent toxicity of Pb, Cu and Ni on *B. japonicum* growth. Even though the bacterial growth was retarded at very low Pb concentrations, the growth was increased under high concentrations. It is possible that *B. japonicum* can develop resistance to Pb by adsorption via extracellular polysaccharides, cell exclusion, sequestration as insoluble phosphates, or ion efflux to the cell exterior (Dopson et al., 2003; Jarosławiecka and Piotrowska-Seget, 2014). As the Ni concentration increases, the growth of *B. japonicum* was inhibited. Similar inhibitory effects on microbes have been documented previously (Babich and Stotzky, 1982). Our results also showed that the growth of *B. japonicum* is reduced in the presence of Cu; such inhibitory effects of Cu on microbes have been documented (Ochoa-Herrera et al., 2011). It has been observed that Zn nano particles increase IAA production in *Pseudomonas chlororaphis* while Cu nano particles decrease IAA production by the microbe (Dimkpa et al., 2012). The IAA production by *B. japonicum* in the presence of Pb, Cu and Ni showed that Pb and Cu do not have an inhibitory effect on IAA production, whereas Ni does. Similar results are reported by siderophore-producing *Streptomyces* spp., which hinder the metal-induced inhibition of auxin synthesis (Dimkpa et al., 2008). Also, this correlates well with the results obtained with turbidity values for Pb. Both bacterial growth and IAA production in the presence of Pb and Cu may have resulted from biofilm formation, to overcome the negative effect caused by heavy metals as reported for the stress-induced production of biofilm in the hyperthermophile *Archaeoglobus fulgidus* (LaPaglia and Hartzell, 1997). Similarly, Pb accumulation by *Burkholderia cepacia* biofilms was reported by Templeton et al. (2001).

The inhibition of seed germination and seedling growth we observed may be due to heavy metal toxicity (Gajewska et al., 2006). The inhibitory and toxic effects of different heavy metal on the germination of seeds and the growth of seedlings are well-documented (Siddiqui et al., 2012; Sethy and Ghosh, 2013). Our results confirm that Ni, Cu and Pb negatively influence lettuce seedling growth. Ni is the most toxic metal of the three we tested, resulting in the lowest root and shoot elongation and highest seedling mortality under high concentrations. Nickel-induced growth inhibitory effects are well-documented (Whitacre and Gunther, 2008) and root inhibition by Ni has been attributed to hydrogen peroxide production (Gajewska et al., 2006). Cu was second to Ni in toxicity, causing reduction in root growth. A study on the effects of soil Cu on black bindweed showed an increase in mortality of the seedlings with the increase of Cu concentration, reaching 40% at 391 mg Cu kg⁻¹ (Kjaer et al., 1998). However, in our study, Pb showed little effect on seedlings compared to Ni and Cu. Nicholls and Mal (2003) showed that Cu can cause more negative effects on plant growth than Pb. It may be due to the root destruction and corresponding retardation of shoot growth in the presence of Cu. However, even though Pb causes shoot inhibition, it causes less damage on roots compared to Cu. Hence roots are able to regenerate in the presence of Pb (Nicholls and Mal, 2003). Previous research suggests that the root growth is more sensitive to metals than shoot growth and seed germination (Araújo and Monteiro, 2005). Our study confirms this trend, showing that root inhibition is more considerable compared to shoot inhibition.

The results obtained in this experiment indicate that inoculation with *B. japonicum* appears to be effective in protecting plants from growth inhibition caused by metals, as strongly supported by the root and shoot length data. This growth promotion may be due to the production of IAA by *B. japonicum* and inhibition of stress ethylene formation. Plant growth promotion by bacteria in the presence of heavy metals is a well-documented phenomenon (Burd et al., 1998; Belimov et al., 2007; Ahemad and Kibret, 2014). However, the exact mechanism of stress tolerance and growth promotion may differ

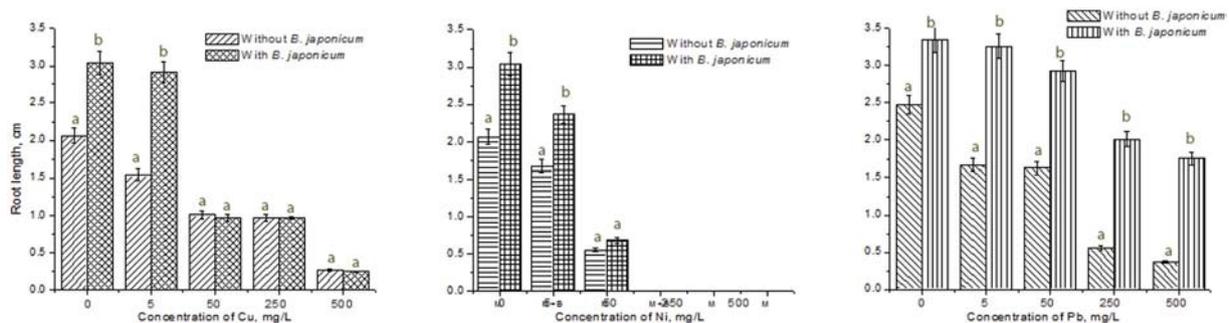


Fig. 5. Effect of *B. japonicum* on root growth of *Lactuca sativa* seedlings under Pb, Cu and Ni. Different letters indicate significant differences ($p = 0.05$, $df = 8$). Error bars represent the standard error of the mean.

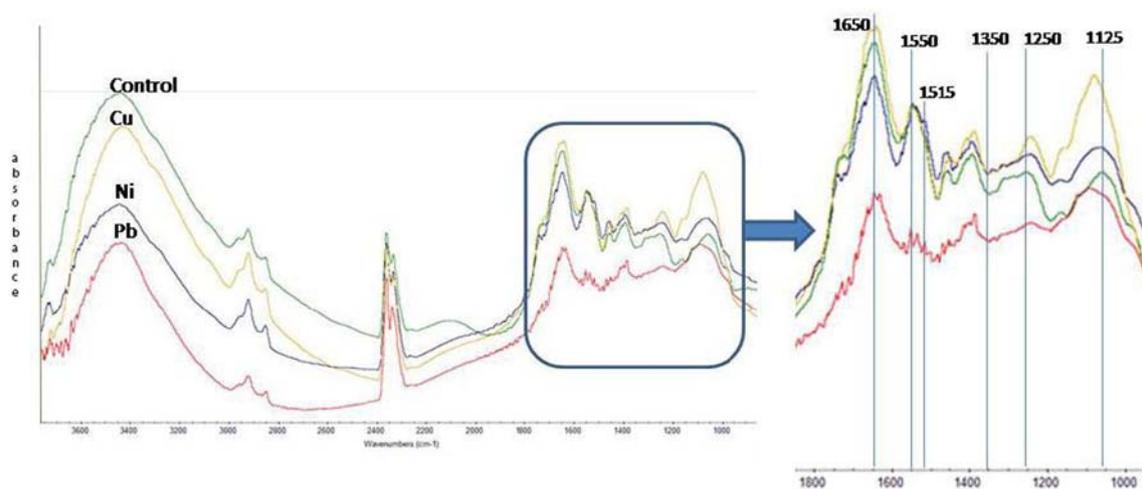


Fig. 6. FTIR spectrum of metal loaded and unloaded biomass of *B. japonicum*.

based on the metal under consideration and the bacterium involved. Burd et al. (1998) reported that *Kluyvera ascorbata* SUD165 was able to enhance plant growth under Ni stress. They suggest that the bacterium protects the plant against the inhibitory effects of Ni-induced stress ethylene.

It is known that contamination of soil with heavy metals increases the accumulation of ACC and ethylene in plant roots (Pennazio and Roggero, 1992). This increased accumulation of ACC facilitates the colonization of rhizosphere with some metal-resistant forms of ACC-utilizing bacteria. These bacteria reduce the stress ethylene production by secreting the enzyme ACC deaminase. In addition to ACC utilization, the metal resistant bacteria offer many other beneficial effects on the host plant. They are: synthesis of siderophores which can solubilize and sequester Fe from the soil (Glick et al., 1998), production of phytohormones which can enhance the growth of plants (Glick et al., 1999), and solubilization of phosphate (Gupta et al., 2002). Glick et al. (1999) reported that IAA produced by plant growth promoting bacteria can enhance root growth by stimulating plant cell elongation or cell division.

FTIR analysis of *B. japonicum* biomass, with and without metal, showed that different functional groups are responsible for the metal ion biosorption process. A broad and strong band in the region of 3200–3600 cm^{-1} and 3500–3700 cm^{-1} could be ascribed to the presence of H bonded and free O–H of alcohol (Pagnanelli et al., 2012). Whereas the presence of the strong peak at 1050–1150 cm^{-1} indicates the C–O stretch of alcohol. The medium peak at 3300–3800 cm^{-1} indicates the N–H stretching, and peaks at 1080–1360 cm^{-1} and 1550–1640 cm^{-1} are respectively due to C–N stretching and N–H bending vibrations of amine (Park et al., 2005). Variable peak region in 1350–1480 cm^{-1} exhibits the C–H bending in alkane. Two strong bands in 1515–1560 cm^{-1} and 1345–1385 cm^{-1} indicate the presence of nitro groups in the bacterial surface. The variable peak region in 1620–1680 cm^{-1} is due to the presence of alkenes. The FTIR spectra of heavy metal-loaded bacterial biomass, in the range of 500–3900 cm^{-1} , were suggestive of the presence of functional groups that are usually responsible for the biosorption process. An obvious change in the peak position and intensity at 1700–500 cm^{-1} region could be assigned to the formation of intense d(M–O) and d(O–M–O) bonds (M = metal ion). Narrowing of and increasing of the height in the peak at 1000–1100 cm^{-1} in Cu-loaded bacterial biomass show the contribution of alcoholic OH on Cu biosorption. Also, the sharpening and shifting of peak at 1200–1300 to 1241 cm^{-1} suggest a strong interaction of Cu with amino groups. A new peak formation was observed in Ni-treated biomass in the region of 1080–1360 cm^{-1} and, the increase in height of peak at 1345–1385 cm^{-1} , indicated the contribution of amine and nitro groups for Ni sorption. Shifting of peak at 1256 cm^{-1}

to 1241 cm^{-1} also indicates the contribution of amine groups for Ni sorption. The FTIR spectrum of Pb-treated *B. japonicum* biomass showed significant changes than in the other spectra. The peak disappearance seen in 1200–1300 cm^{-1} region and at 1450 cm^{-1} and the reduction in peak height at 1545 cm^{-1} shows the role of amine and nitro groups in Pb biosorption. According to the FTIR spectra the amino, alcohol and nitro groups are responsible for Cu, Ni and Pb biosorption processes. FTIR results showed that Pb was more favorably adsorbed than Cu and Ni. The strength of adsorption of Ni, Cu and Pb on the bacteria is in the order of Pb > Cu > Ni. It reflects that Ni is the least adsorbed and Pb is the most adsorbed with *B. japonicum*. The bacterial growth changes under Ni, Cu and Pb stress can be due to the differences in adsorption of these metal ions. The growth reduction of bacteria with Ni and Cu may be due to lower adsorption of these ions by the bacterial cell wall, contributing to greater uptake of metals into the bacterial cell, causing growth inhibition. The growth of bacterial cells even under high Pb concentrations may be due to adsorption of Pb by the bacterial cell wall.

5. Conclusions

The results revealed that Cu and Ni can cause reduction in growth of both *B. japonicum* and lettuce seedlings. However, Pb did not show a considerable negative effect on *B. japonicum* and lettuce seedlings within the experimental range. The increased bacterial growth under Pb may be due to the sorption of Pb by the bacterial cell wall. IAA production by *B. japonicum* was also affected by heavy metal stress. It follows the same pattern as that for growth under Pb and Ni, whereas there was no reduction in IAA production under Cu. According to the results we obtained in the lettuce seedling assay, *B. japonicum* is able to promote plant growth under low concentrations (5 ppm) of Cu and Ni whereas it is successful in promoting plant growth up to 500 ppm of Pb.

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