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Research Article

EFFECT OF PLANT GROWTH PROMOTING RHIZOBACTERIA ON PLANT GROWTH UNDER LEAD AND CADMIUM STRESS CONDITION : A STEP TOWARD SUSTAINABLE AGRICULTURE

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ABSTRACT

The contamination of soil and water by different heavy metals are the burning problem now a days and it is escalating day by day due to excessive industrialization, agricultural applications, various anthropogenic sources, waste disposals etc. Heavy metals like Cadmium and Lead retard the vigor and growth of plants and also produce serious health problems of humans. To avoid the problem caused by Cadmium and Lead can be overcome by using Plant Growth Promoting Rizobacteria (PGPR) which can easily grow under those heavy metal condition. We isolated some Cadmium and lead resistant PGPRs from expected heavy metal contaminated rhizospheric soil. Out of these bacteria two best PGPRs were exploited for plant growth under in vitro Cadmium and Lead polluted condition. The results showed that these two PGPRs enhance the plant growth and vigor under Cadmium and Lead stress condition.

INTRODUCTION

In many developing countries, agriculture is the major portion for the national economy. Development of improved plant varieties and different technological interventions fulfill the demands of the growing population in the country (Johri, 2003). During the past few decades high yielding varieties, chemical fertilizers and pesticides produces huge revolution in agriculture. Although the chemical fertilizers and chemical pesticides has several advantages like simplicity of handling and predictable results but there are also some problems regarding application of chemical fertilizers to the soil. They sows deleterious effect on soil ecology, high irrigation needs, as well as hamper the human health (Harman 1992). Excessive use of chemicals caused deterioration in the chemical, physical, and biological health of cultivable land (Paroda 1997). In the natural ecosystem chemical fertilizer caused environmental hazard, pollution and changes. Sustainable agriculture has the potential to meet our future agricultural needs so it is very important in today's world. Wastewater irrigation is the main source of heavy metal contents of soils (Mapanda, 2005). Some plants can accumulate various heavy metals and when these plants are consumed as food stuff, causing a serious health risk to humans (Wenzel, and Jackwer.1999) . Industrial waste and sewage water disposal is a great problem as these water contained not only organic matter but also different heavy metals like Fe, Mn, Cu, Zn, Pb, Cr, Ni, Cd and Co as well (Kabata-Pendias, 2001). Often it is drained into agricultural land used for many vegetable and other crops. Heavy metals present in many foodstuffs to certain concentration can cause serious disruption in biological and biochemical processes in the human body, while some heavy metals like arsenic, cadmium, chromium act as carcinogen and mercury or lead produce developmental abnormalities in children (Adriano, 1986). Due to very long half life and wide spread in nature (Chien *et al.*,2002) Cadmium hamper the plant growth directly by disrupting the photosynthesis activity (Zhang *et al.*, 2002). Cd markedly produce hydroxyl radical (OH^-), ROS, H_2O_2 and super oxide anion (O_2^-) in plant body and ultimately it hinder the plant growth (Mobin and Khan, 2007). As vegetable contained many essential vitamins, minerals, iron, calcium and many other functional food components, they are important part of human diet (Arai 2002). Higher amounts of heavy metals are abosorbed by vegetables especially leafy vegetables grown in heavy metal contaminated soil (Jassir 2005).

Rhizobacteria which has the plant growth promoting ability can be used as biofertilizer, phytoremediation and biocontrol agents for increasing plant vigor and soil fertility (Dastager, 2011). Application of PGPR is an attractive alternative for environmental pollution causing chemical fertilizers (Ali, Sabri and S. Hasnain 2010). PGPR which has the heavy metal resistant ability can survive under heavy metal contaminated soil as well as they promote plant growth by directly or indirectly by Phosphate solubilization, Indol acetic acid

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production, Nitrogen metabolism etc (Perveen, Khan, and Zaidi 2002).

In the current scenario the objective of study is the improvement of crop growth and yield in heavy metal stress condition by exploitation of PGPR by use of spinach as test plant.

MATERIALS AND METHODS

Collection of Soil Sample: Five different rhizospheric soil samples were collected from agricultural land which were polluted with industrial effluent water around Uluberia [Howrah district, West Bengal]. 4 to 5 week old plants were uprooted and the soil samples adhearing to the root surface were collected in clean polythin bags brought to the laboratory, and kept in dry , aseptic condition for further use.

Isolation of Bacteria: Bacterial strains were isolated from collected soil samples by standard soil dilution plate count technique used nutrient agar as supporting medium [Peptone – 5.0 ,Beef extract – 3.0 , Agar – 15.0 , NaCl – 5.0 , pH -7.0 , Water – 1 liter]. The plates thus prepared were incubated at 37^0 C for 3 days and after 24 hr interval plates were observed for the growth of bacteria

Determination of minimum inhibitory concentration (MIC) of Cadmium (Cd) and Lead(Pb) on the isolated bacterial strains: Nutrient agar medium supplemented with different concentrations (Such as- 50,100,150,----------600 ppm) of Cadmium or Lead were prepared, plated and then streaked with different individual isolated bacteria. The plates were incubated at 37˚c ± 2˚c for 48 hrs. and bacterial strains developed if any, were recorded and pick up on slant for future work.

Characterization of Bacteria: The selected bacterial isolates were characterized by their colony morphological , Gram staining and biochemical properties. Colony morphological characters including color, elevation and edge of the colony. Gram nature of each isolates was initially determined by using crystal violet and safranin staining

Biochemical Characterization: For biochemical characterization of isolated bacteria following standard tests such as Catalase test, Amylase test, Gelatin hydrolysis test, Methyl red test, Indol production test, Citrate utilization test, Casein hydrolysis test and Urease test were performed.

Determination of plant growth promoting (PGPR) ability:
Detection of Phosphate solubilized ability: Phosphate solubilizing ability, were determined following the isolate bacteria by standard microbiological technique. The bacteria isolates were inoculated into the sterile petriplates containing pikovskayas medium (HI media) and incubated for 2 - 3 days at 37±2˚c after incubation, observed the hallow zone production around the colony for the positive result.

Detection for the Ammonia Production: Amonia production was determined by the method of Dye (1962) . Each selected bacterial isolates were inoculated in the peptone water broth (Peptone -4.0 g ,Water – 1 liter, pH -7.2) and left for 4 days in 37 ˚c±2˚c. after incubation period ,1 ml of Nesslers reagent was added to the tubes. Change of color to deep yellow brown of the broth indicates the ability of each isolates for ammonia production.

Detection for IAA production ability- Each bacterial strains were inoculated to 20 ml of Luria Bertani (LB) (Tryptone 10g, Yeast extract 5g, NaCl 10 g, Agar 20g Distilled water 1 lit.,PH 7) supplemented with L-tryptophan (0.2%) and incubated in 24^oc rotary shaker then centrifuged at 10000g for 15 min. 2 ml of supernatant was collected and 2-3 drops of O-phosphoric acid along with 4 ml of salkowski's reagent added (100 ml of 35% of perchloric acid along with 2 ml of freshly prepared 0.5 M $FeCl_3$ solution). Then incubated for 30 min in dark room. Absorbance were recorded in 530 nm. Quantity of auxin were calculated from the standard curve using indol acetic acid as standard (10-100µg).

Detection for HCN production ability- Hydrogen cyanide (HCN) production ability was observed as per methodology described by Bakker and Schipperes method (Bakker W & Schippers B,1987). Bacterial cultures were inoculated on 4.4 g/lit of glycine containing king's B agar medium. A Whatman No 1 filter paper soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed inside the lid of a plate under sterile condition. Plates were properly sealed and incubated at 30±0.1°C for 4 days. Production of orange to dark brown color shows HCN production.

Collection of seed: Spinach (*Spenacia oleracia* var. harita) Seeds were collected from seed collection centre of Bidhan Chandra Krishi Viswavidyalaya,W.B. India.

Seed germination test: The spinach seeds were surface sterilized with 0.1% $Hgcl_2$ for 3 minutes followed by with dilution of culture medium for 24 hrs. After 24 hrs. the seeds were placed on the sterile bloting paper. Different concentration (Cd-30ppm or Pb-60ppm) and Combination of heavy metals (Cd+Pb) were added to these petri plates. Seeds were treated only with distilled water in case of control sets. Number of seed germination were recorded after 24 hrs. intervals upto 6 days. To determine the effect of bacterial inoculants on seed germination in different treatment was determined by apply to different combination of bacterial culture as mentioned below. Total 15 experimental set up and a control set up containing without HM and sterile seeds has been established.

Control (No. Bacteria + No. HM)	C	P	CP
Only S-2-3-4	C+ S-2-3-4	P+ S-2-3-4	CP+ S-2-3-4
Only S-2-4-4	C+ S-2-4-4	P+ S-2-4-4	CP+ S-2-4-4
S-2-3-4+ S-2-4-4	C+ S-2-3-4+ S-2-4-4	P+ S-2-3-4+ S-2-4-4	CP+ S-2-3-4+ S-2-4-4

The concentration of cadmium used for this study was 30 ppm concentration of Lead was 60 ppm and concentration of cadmium and lead in the Experimental Set up where these 2HM are jointly applied were Cadmium (C) →30 ppm and Lead (P)→ 60 ppm.

Pot Experiments: One third depth of pot were filled with sand and upper remaining portion were filled with fine sleeved soil. Soil and sand were previously sterilized with autoclave at 15 lb

pressure for 1 hour. Spinach seeds (Variety: Harita) were imbibed selected in nutrient broth inoculated with the selected bacteria separately and in the other Set 2 bacteria are inoculated combinely as mentioned previously. These imbibed seeds were shown on the different pots containing different treatments with one control set up as mentioned earlier during the seed germination test. All the experimental set up were triplicated.

After 14 days of growth the Spinach sapling were uprooted carefully and observed the shoot length (cm) , root length (cm), fresh weight(mg), dry Weight(mg) were recorded. Chlorophyll content (mg/gm of tissue) were recorded spectroscopically (Microprocessor visible Spectro photometer, model no-LI-722, Lasany. Made in India) of different plant sample obtained from different experimental set with respect control set.

RESULTS AND DISSCUSSION

In the study the bacterial load of 5-different agricultural soil contaminated with industrial effluent was estimated. It was noted the numbers of bacteria obtained in different soil samples was variable perhaps due to intrinsic ecological properties of soil. It was further noted that the results that the resistance on cadmium and Lead of the test bacterial isolates was also variable. Some of the isolates (S-2-4-4,S-2-3-4,S-1-6-5,S-1-2-4 and S-2-2-4) even had the tolerance level upto 150ppm and 600ppm respectively for long time cd & Pb contaminated soils (Table no 1). Which gradually accustain the bacteria to grew in such a contaminated condition. Similar type of results previously obtained and the data therefore in this study was in confirmation with the previous finding (Ma *et al* 2009). In a separate experiment the morphology, Gram nature , and biochemical properties of the isolates were enumerated . It was noted that all the five test isolated were variable in their characteristic features. The distinct morphological characters (Table no 1) followed by Gram nature and biochemical tests (Table no 2) indicated that the isolates are different strains and although had few common characters further indicated they might be close in taxonomic status. Among the isolated bacterial strains 5 were selected according to their ability to tolerate Cadmium and Lead stresses. Out of 5 strains, 2 strains (S-2-2-4 and S-1-2-4) has tolerance upto 500 ppm of Lead and 2 strains (S-2-4-4 and S-1-6-5) has 400ppm of lead tolerance. S-2-3-4 has the greater ability to tolerate upto 600 ppm. All the isolates has the tolerance up to 150 ppm of Cadmium stress.

Table 1 Selected bacterial strains on the basis of Cadmium (Cd) and Lead (Pb) tolerance with their colony morphology

Bacterial strains	Colony morphology	MIC of Cadmium (ppm)	MIC of lead (ppm)
S-2-4-4	Creamish, round ,smooth, flat	150	400
S-2-3-4	White, round , rough, flat, elevated margin.	150	600
S-2-2-4	White, round, rough, raised, elevated margin	150	500
S-1-2-4	White, serrate margin, raised, undulated.	150	500
S-1-6-5	Yellowish, irregular, lobately margin	150	400

Among the 5 isolates 2 were Gram positive and the rest 3 were Gram negative. Different biochemical tests are depicted in the Table no 2. Among the biochemical tests (Catalase test, Amylase test, Gelatin hydrolysis test, Methyl red test, Indol production test, Citrate utilization test, Casein hydrolysis test and Urease test) positive results has been observed of all the isolates in case of catalase and gelatin hydrolysis Indol production test. 2 strains out of 5 shows positive result in amylase test.

Different plant growth promoting ability are depicted in the table no 2. In case of ammonia production test, all the selected isolates showed positive result but in case of phosphate solubilization and IAA production test only S-2-4-4 shows positive results. But in HCN production test all the 5 selected bacterial strains produced negative result. From these PGPR confirmation tests it can be concluded that S-2-4-4 have the best plant growth promoting ability.

Plant response analysis test

Seed germination test- In the germination test (Figure 1) of spinach seeds indicated that the heavy metals significantly inhibit the germination. In case of heavy metal treatment in this study depicted that the heavy metals have some adverse effects on seed germination and finally reduce the ability of germination than the control set where no heavy metals were added. Somehow the selected bacteria were able to reduce the deleterious effects of heavy metals appreciably. Lead treatment reduced the seed germination than the Cadmium-30ppm treatment. Though Cadmium radically decreased the seed germination rate than the control set. But inoculation of S-2-3-4 and S-2-4-4 produced a significant enhancement in the percentage of germination where seeds were embedded in Cd-30 and Pb-60 ppm solution. Similar kind of observations were noted by Pal *et al* (2004).

Determination of root / shoot length (cm)

It was observed that the treatment of Cd, Pb and combination of Cd+Pb significantly reduced root length and shoot length (figure 2 and 3). Root and shoot length were decreased respectively by 56.3% and 15.9% under Cadmium stress, 5.9% and 23.5% under combined heavy metal stress. But when S-2-3-4 or S-2-4-4 bacteria are applied the root and shoot length were significantly increased. Among these 2 bacteria apparently S-2-4-4 produced better growth enhancement under heavy metal stresses .

Under lead stress condition S 2-3-4 enhanced root length and shoot length respectively by 78.8%, 0.3% and S-2-4-4enhanced 54.1% and 31.61% than the root length and shoot length under lead stress without PGPRs. Similar type of growth enhancement were observed under lead or combination of Lead and Cadmium stress condition by using isolated PGPRs. The data therefore in confirmation of the previous observation made by several workers (Aydinalp and Marinova, 2009; Rajkumar and Freitas, 2008) with different bacteria and different test plants in separate ecological condition.

Table 2 Characterization of bacteria

Bacterial strains	Gram nature	Catalase test	Amylase test	Gelatin hydrolysis test	Methyl red	Indole production	Citrate utilization	Casein hydrolysis	Urease test	PGPR confirmation test			
										Phosphate solubilization test	Amonia production test	HCN production	IAA production
S-2-4-4	+ ve rod	+	-	+	+	+	-	++	-	++	++	-	++
S-2-3-4	-ve rod	+	+	+	+++	+	-	-	-	-	+	-	-
S-2-2-4	-ve coccus	+	+	+	++	+	+++	+++	-	-	+	-	-
S-1-2-4	-ve coccus	+	-	+	+	+	-	-	-	-	+	-	-
S-1-6-5	+ve rod	+	-	+	+	+	-	-	-	-	+	-	-

(‘+’ or ‘-’ sign indicate the positive or negative approach of the test. No of ‘+’ sign denote the intensity of yellow colouration, bubble production or liquefaction of gelatin respectively for amylase, catalase and gelatin hydrolysis test.)

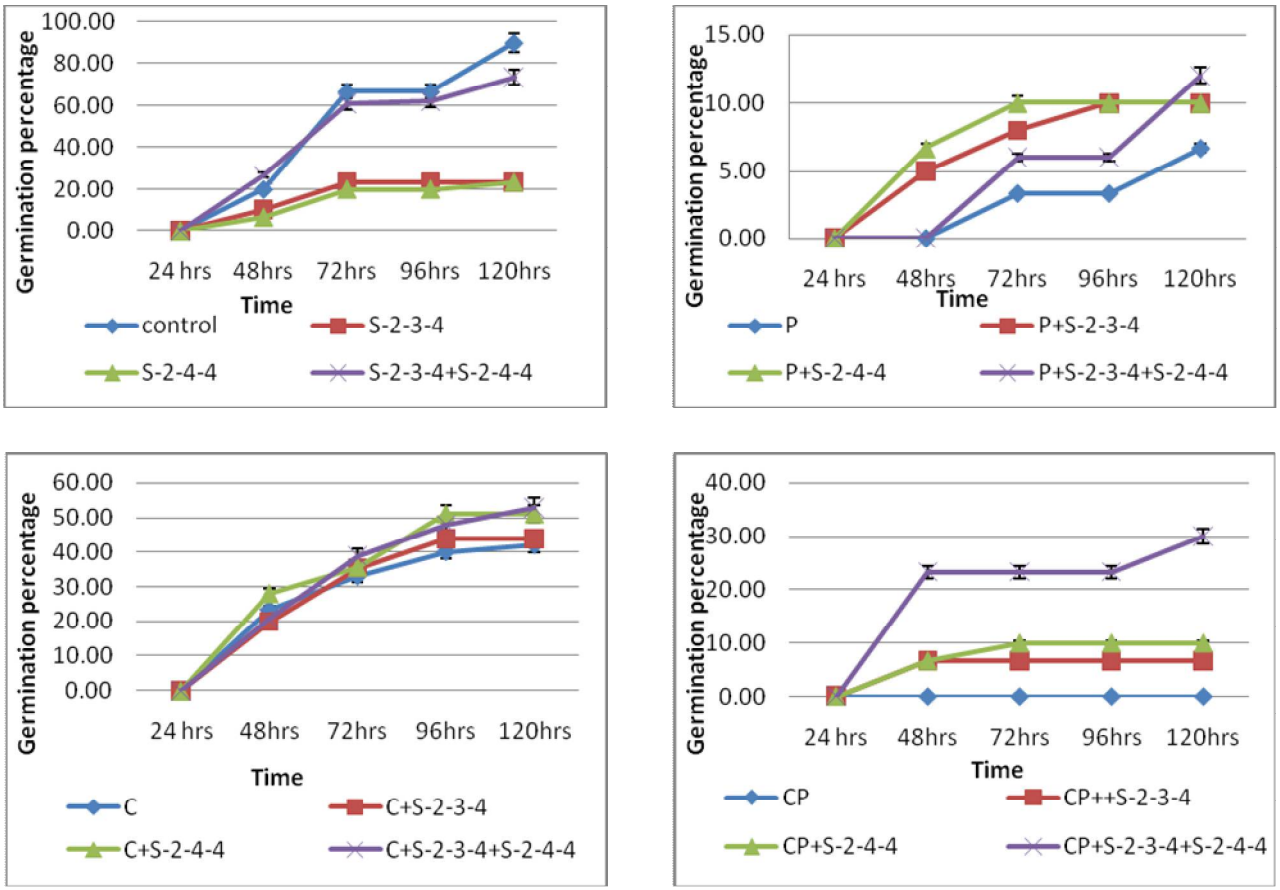

Figure 1 Depicted the effect of 2 bacterial isolates (S-2-3-4 and S-2-4-4) in Cd and Pb stress on germination rate of spinach seed

Figure 2 Showing comparative analysis of Cd & Pb with two selected Bacterial strain on Root length and Shoot length On Spinach seedling.

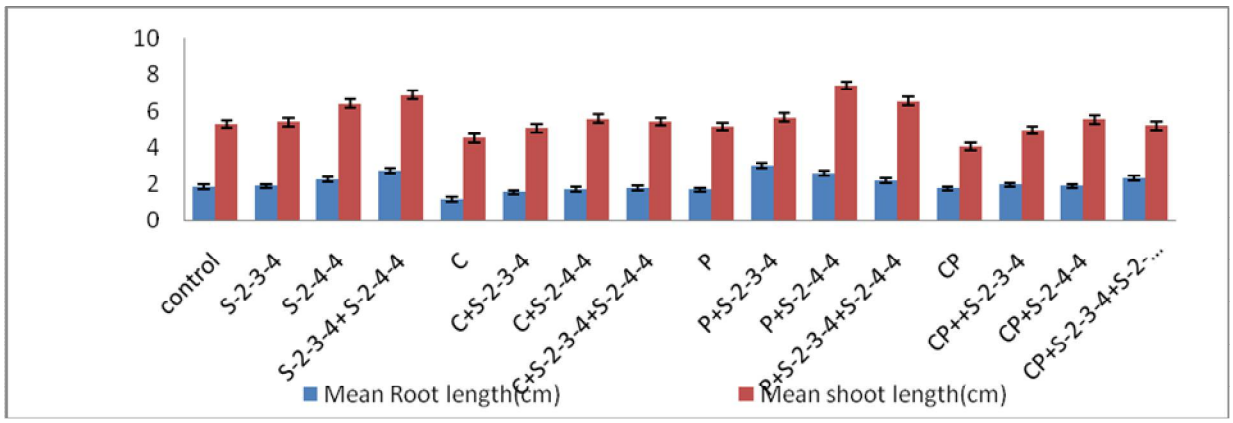

Figure 3 Depicted the effect of 2 bacterial isolates (S-2-3-4 and S-2-4-4) in Cd and Pb stress on root length and shoot length of spinach seed

Estimation of fresh weight and dry weight- Both fresh weight and dry weight were significantly increased when treated with the selected plant growth promoting bacterial strains under heavy metal stress conditions (figure 4). The results further indicated that both fresh weight and dry weight of spinach were markedly decreased due to the adverse effects of heavy metal. Here also in presence of PGPR the heavy metal stress condition were markedly nullified and promote the growth. The results obtained therefore support the previous observation made by Kamran *et al* (2015) in case of *Eruca sativa* where inoculation of bacteria caused overcome the adverse effect of heavy metal.

Estimation of chlorophyll content

Chlorophyll content was significantly decreased after heavy metal treatment especially under Cadmium stress than the control set (figure 5). It may be due to the effect of Cadmium which inhibits the chlorophyll biosynthesis. These results confirm the previous results observed by Somashekaraiah *et al.*(1992). Both the selected PGPRs certainly nullify the deleterious effects of heavy metal in case of chlorophyll biosynthesis. Zhang *et al.* (2011) also observed that PGPR can enhance the chlorophyll content under Cadmium stress condition.

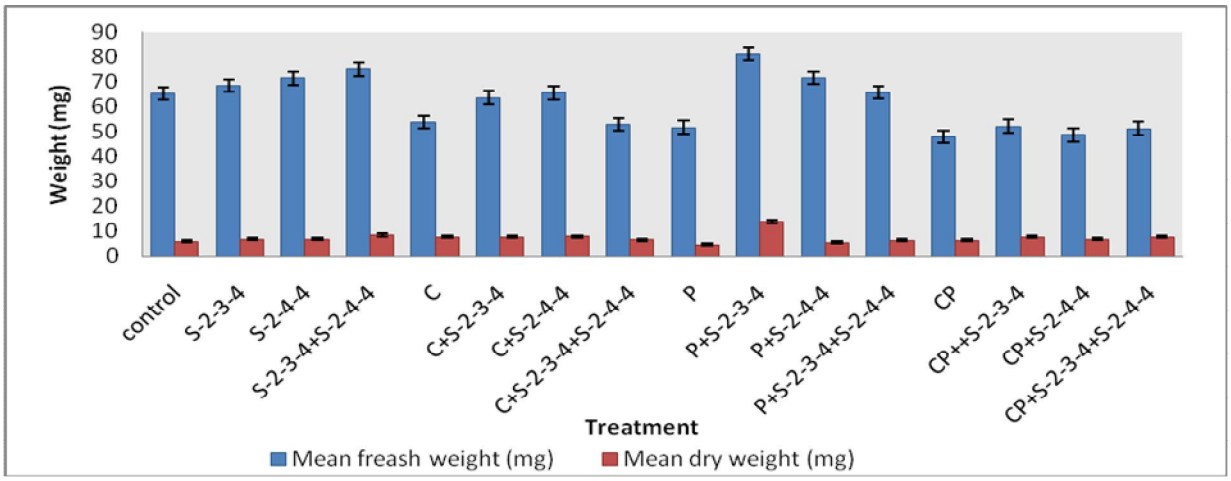

Figure 4 Depicted the effect of 2 bacterial isolates (S-2-3-4 and S-2-4-4) in Cd and Pb stress on fresh weight and dry weight of spinach seed.

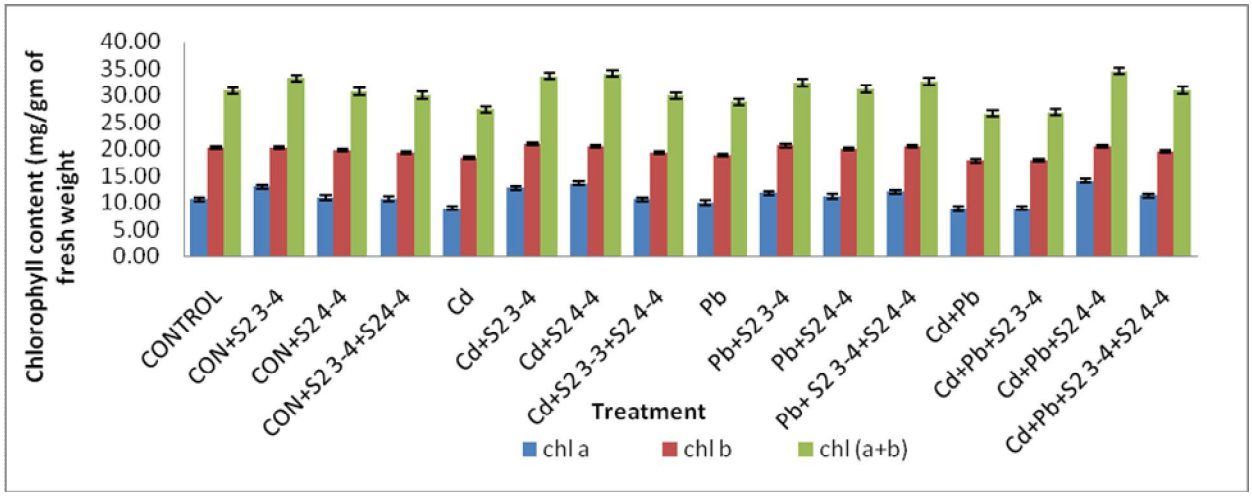

Figure 5 Depicted the effect of 2 bacterial isolates (S-2-3-4 and S-2-4-4) in Cd and Pb stress on chlorophyll content of spinach seed.

CONCLUSION

In general it was noted that heavy metal stress markedly reduced different growth parameters such as the root length, shoot length and chlorophyll content of spinach plant. The adverse effects of these stresses were remarkably improved by inoculating plant growth promoting rhizobacteria which gave an indication that Suitable PGPR strains may be use for betterment of plant growth in normal agricultural as well as in such heavy metal stress condition. Moreover its proper doses may be essential for such practice whether plant species and agro-climatic condition may play a vital role in such study. This is a preliminary observation and further study need to develop this practice for establishment in commercial basis.

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