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Phyto-extraction of chromium and influence of plant growth promoting bacteria to enhance plant growth

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ABSTRACT

Chromium (Cr) toxicity affects many physiological processes and inhibits plant growth. Plant growth promoting rhizobacteria (PGPR) have ability to improve plant health in contaminated soil as well as degrade toxic Cr. Present investigation was made to examine the role of *Pseudomonas putida* (ATCC 39213), to enhancing the plant growth and Cr phytoextraction. Three treatments of Cr (low; 150 ppm, medium; 250 ppm and high; 500 ppm) were applied to the *E. sativa* seedlings, either inoculated with *Pseudomonas putida* (*P. putida*) or un-inoculated plants. Results of this study clearly indicate that *P. putida* inoculation significantly increased plant growth and Cr uptake as compared to un-inoculated plants. Root and shoot length increased 33% and 42%, fresh and dry weight increased 49% and 53% as compared to un-inoculated plants, respectively. However, chlorophyll and proline contents increased 39% and 44%. Physiological parameters of growth indicate that Cr toxicity decreased the plant growth, while inoculation of *P. putida* overcome the inhibitory effects of Cr and increased growth under Cr stress. Cr uptake by plants was increased 38% with *P. putida* inoculation. Provision of Indole acetic acid, siderophore and 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity by *P. putida* in the growing media, which may induce the Cr uptake and plant growth under stress condition in *E. sativa*. The present results offer insight on plant growth promoting rhizobacteria's (PGPRs), such as *P. putida*, potential to enhance the plant growth by inhibiting the adverse effects of Cr in *E. sativa*. This study will contribute towards the environmental management of Cr-contaminated areas and enhancing plant growth under Cr stress conditions.

1. Introduction

Heavy metals are continuously being added to soils through various agricultural and industrial activities, agrochemicals and the long-term deposition of urban sewage sludge on agricultural soils, waste disposal, waste incineration and vehicle exhausts (Husain et al., 2008; Kamran et al., 2014; Bibi et al., 2015a). All these sources cause accumulation of these elements in agricultural soils and pose a threat to food safety and potential health risks among heavy metals, Cr is relatively mobile in soils and is one of the most toxic. In plants, Cr inhibits root and shoot growth, affects nutrient uptake and homeostasis, and is frequently accumulated by important crops consumed by animals and humans (Abdullah et al., 2015). Contamination of soil with Cr also negatively affects biodiversity and the activity of soil microbial communities (Kamran et al., 2014).

In recent years considerable interest has been developed towards the phytoremediation, to treat heavy metal contaminated soils (Boopathy, 2000; Glick, 2010). Phytoextraction, the absorption and accumulation of metals from soil to roots and shoots (Burd et al., 2000), with its in situ, lower cost and environmental friendly nature, is considered as a novel and highly promising technology for metal-polluted sites remediation. However, phytoextraction process suffers from several main limitations: (a) low bioavailability of heavy metals in the soil, (b) low translocation rate of metal from roots to shoots and (c) low biomass of the applied plants. This may further extend the time needed for soil clean-up, which is generally relatively long (up to several decades) in phytoextraction systems. Extensive research has been conducted on process optimization by means of chemically improving plant availability and uptake of heavy metals (Evangelou et al., 2007).

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However, those synthetic compounds such as EDTA which assists phytoextraction have received increasing criticism due to their environmental persistence and associated risks for leaching (Bibi et al., 2015b). A promising alternate method for enhance phytoextraction efficiency is carried out by bioaugmentation (Lebeau et al., 2008). Special attentions have been paid on the plant growth-promoting bacteria (PGPB) among the rhizosphere microorganisms involved in plant interactions with the soil environment. Previous studies showed that certain bacteria could alleviate heavy metals toxicity for plants and promote plant growth (Burd et al., 2000; Rajkumar and Freitas, 2008; Dell' Amico et al., 2008). On the other hand, some metal resistant microbes with plant growth-promoting (PGP) properties could improve the availability of heavy metals for plant uptake (Abou-Shanab, et al. 2006; Sheng and Xia, 2006). Soil microbes help in different reactions and metabolic processes taking place in biogeochemical cycles of nutrients, soil structure maintenance, detoxifying pollutants and production of essential compounds for both microorganisms and plant (Khan et al., 2010; Kamran et al., 2015). Production of phytohormones and bacterial assisted phytoremediation of heavy metal have great importance for decontamination of polluted environment (Bibi et al., 2008; Hao et al., 2012). Plant growth promoting rhizobacteria (PGPR) exert beneficial effect on plant growth. Symbiotic microbes associated with plants are also involved in providing nutrients and reducing the toxicity of metals to the plants. By abating the toxicity of heavy metals to the plant, phytoredmediation could be enhanced. However, PGPR enhance heavy metal stress tolerance by detoxification of metals absorbed (Glick, 2003; Han et al., 2005; Tassi et al., 2008). Chen et al. (2010) reported that *Cupriavidus taiwanensis* is an indigenous metal resistant PGPR bacterium that makes a symbiotic association with *Mimosa pudica*. This association is very beneficial for the removal of heavy metals, because free living *M. Peduca* absorb fewer amounts heavy metal (Pb, Cu, and Cd) as compared to nodulated (of *C. taiwanensis*) which absorb higher amount of heavy metal. As *P. putida* (gram-negetive) has various mechanisms of metal resistance and homeostatic regulation of several metals and metalloids, it is adapted to grow in metal contaminated environments (Kowalski, et al. 2002; Yousaf, et al. 2010). However, *P. putida* colonize plants roots in rhizosphere and improve plant growth (Ma et al., 2011). *P. putida* assist in promoting plant development and growth therefore, researchers use this bacterium in bioengineering research to produce biopesticides consequently improving plant health (Kamran et al., 2016). In this study, we used *Eruca sativa* to remediate the Cr pollution in the soil, as it has fast growth rate and can survive in different climatic conditions through the region. In Pakistan, Chromium is widely used in electroplating, leather tanning, textile dyeing, and metal processing industries (Zafar et al., 2015), which can be remediated by the cultivating *E. sativa*. We assessed the influence of PGPR (*P. putida*) to enhance the capacity of Cr accumulation and tolerance assisted by the plant *E. sativa*, a Cr hyperaccumulator using pot trial experiment approach. The results of the present study would be very useful to deal with the worst situation of Cr contamination at wide scale industrial areas and ultimately pose several health risks to both wildlife and human.

2. Material and methods

2.1. Soil preparation

Soil samples were collected from a depth of 20 cm from non-contaminated fields. All the samples were air-dried, sieved (through a sieve of 2-mm mesh size) and stored at 4 °C. Total contents of Cr in the soil were measured by using Atomic Absorption spectrophotometer (Varian FAAS-240) by following the method described elsewhere (Malik et al., 2010). Soil physiochemical properties; soil texture, pH, EC and soil organic matter was measured (Amna et al., 2015).

2.2. Characterization of bacteria

2.2.1. ACC deaminase activity

ACC deaminase activity of cell-free extracts was determined by observing the quantity of α-ketobutyrate produced by the enzymatic hydrolysis of ACC (Belimov et al., 2005). The absorbance (OD540) was compared with a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 mol, a method described by Penrose and Glick (2003).

2.2.2. IAA production

Bacterial IAA was measured according to Glickmann and Dessaux (1995). Single bacterial colonies were inoculated into LB medium with or without Cr and grown overnight at 200 rpm in a shaking water bath at 30 °C. The absorbance at 600 nm of each culture was adjusted to approximately 1.2, and 5 ml of each culture was inoculated into 5 ml DF medium supplemented with (NH4)2SO4 and containing L-tryptophan (200 mg ml^{-1}). The cultures were shaken at 200 rpm at 30 °C for 72 h. Cells were harvested by centrifugation at 11,000 rpm for 15 min at 4 °C and 2 ml of Salkowski's reagent was added to 1 ml of supernatant and the IAA content was determined by measuring the OD535 nm.

2.2.3. Synthesis of siderophores

Siderophore secretion by strains was qualitatively detected by the "universal" method of Schwyn and Neilands (1987) using blue agar plates containing the ternary complex chrome azurol S (CAS)/iron (III)/hexadecyltrimethyammonium bromide as an indicator. Change in the dye colour from blue to orange indicated production of siderophore.

2.2.4. Culture preparation and seed inoculation

The bacterial strain was grown on Tryptic yeast extract (Dary et al., 2010) medium and incubated at 30 °C on the rotary shaker at 250 rpm for 24 h, then; it was mixed on an orbital shaker with a speed of 120 rpm and incubated at 25 °C for 72 h (Bhuvaneswari et al., 1980; Hadi and Bano 2010). The incubated broth cultures were centrifuged at 3000 rpm for 15 min. Pelleted cells were re-suspended in sterile tap water and adjusted to about 108 cells ml-1 based on an optical density (OD660 = 0.08) (Hadi and Bano, 2010). Seeds were surface-sterilized by soaking them in ethanol for 1 min, rinsed shortly in 0.1% of $HgCl_2$ and then thoroughly washed then with distilled water according to the method described by El-Abyad et al. (1993). Sterile seeds were incubated for 2 h in 40 ml of bacterial suspension and gently stirred in the dark at room temperature, after which they were removed from the suspension using sterile pliers and sown. Seed sterility was verified by incubating 10 seeds on LB agar at 30 °C for 10 days without any contamination appearing.

2.2.5. Experiment design

Pot experiment was conducted to analyze the effect of *P. Putida* on growth of *E. sativa* and Cr uptake. Three seeds were sown in each pot and six pots were used per treatment. The pots containing seeds were kept in the growth chamber (temperature 27 ± 3 °C), under a light irradiance of 300 μmol m^{-2} s^{-1} (12/12 h light/dark cycles) for 22 days, and Cr dose was applied as treatments (T1–150, T2–250, and T3–500 μg/g), total of 60 ml aqueous solution of Cr levels was applied to the respective pots. After 3 weeks of Cr treatment, the plants were removed, washed and analyzed for different growth parameters, i.e., root length, shoot length, dry and fresh weight, chlorophyll content, proline and metal contents. At the end of experiment, plant samples were collected, divided into shoots and roots and washed with deionized water. To determine the dry weight (mg), roots and shoots (biomass) oven-dried separately at 75 °C. Then samples of roots (0.1 g) and shoots (0.2 g) were digested with a mixture of H_2SO_4/H_2O_2. The Cr concentration (μg/g) was measured by using Atomic Absorption spectrophotometer (Varian FAAS-240). Chlorophyll of leaves was determined by a chlorophyll meter (Minolta SPAD-502 Leaf Chlorophyll Meter) (Mantelin

and Touraine, 2004). For the determination of proline contents in plants, the 0.5 g of plant material homogenized in 5 ml of 3% sulphosalicylic acid and incubated at 100 °C for almost 12 min then 2 ml of glacial acetic acid and 2 ml of ninhydrin was added into supernatant and kept for 60 min at 100 °C. After cooling the mixture 4 ml of toluene was added to mixture. The photometric absorbance of toluene extract was read photo metrically at 520 nm (Bates et al., 1973).

2.3. Statistical analysis

Observations were made and all the experiments run in triplicates. At least three separate flasks were maintained for one treatment. Each time three readings were taken, and mean value and standard error were calculated. Excel software was used to compile all results to form a database. Basic descriptive statistics was calculated for all the growth parameters of all samples. ANOVA was obtained by using Duncan's multiple test in order to have treatments comparison at 0.05 significance level. All of the statistical procedures were performed by using Statistics software (version 8.1).

3. Results and discussion

In present study, we screened out the metal uptake potential of *E. sativa* that were subjected to Cr-contaminated soil and the effect of *P. putida* on the plant growth and Cr accumulation mechanism. We assessed the plant response by *P. putida* inoculated vs. non-inoculated at three different levels of Cr-contaminated soils. We measured the plant growth response by following several parameters Viz; root length (cm), shoot length (cm), fresh weight (g) and dry weight (mg), chlorophyll contents (SPAD) and proline content (μg/g).

3.1. Physical and chemical properties of soil

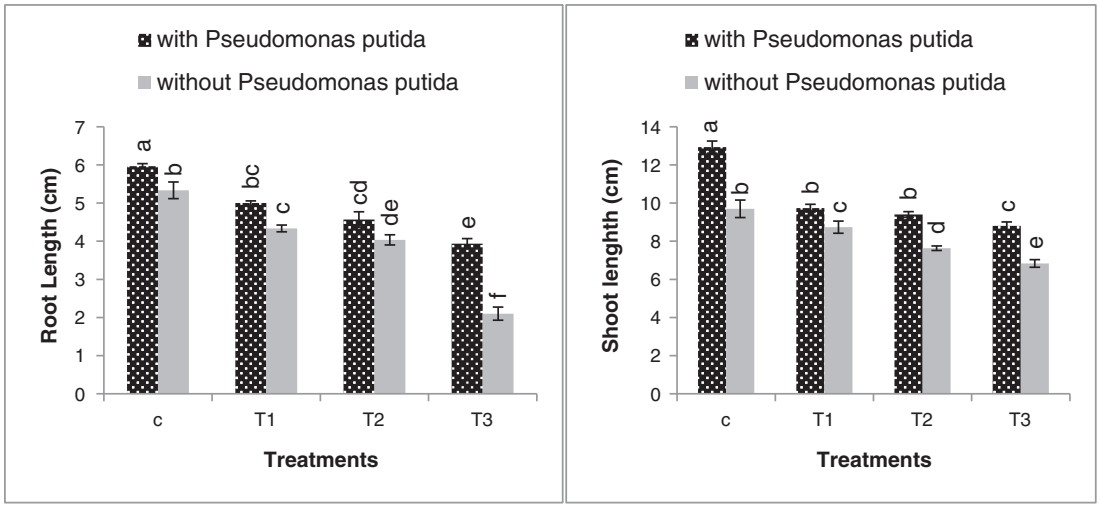
Soil used in the experiment was analyzed for different physical and chemical properties. Results of the analysis showed that the texture of the soil was loam. The pH of the soil was neutral. Soil was calcareous in nature, not efficient in N and low in organic matter.

3.2. Characteristics of the bacteria

The bacterial strain had high Cr-resistance and utilized ACC as the sole source of nitrogen and possessed ACCD which hydrolyses ACC. Siderosphore production of bacteria was positive, while results of Gram's staining showed that isolate was negative.

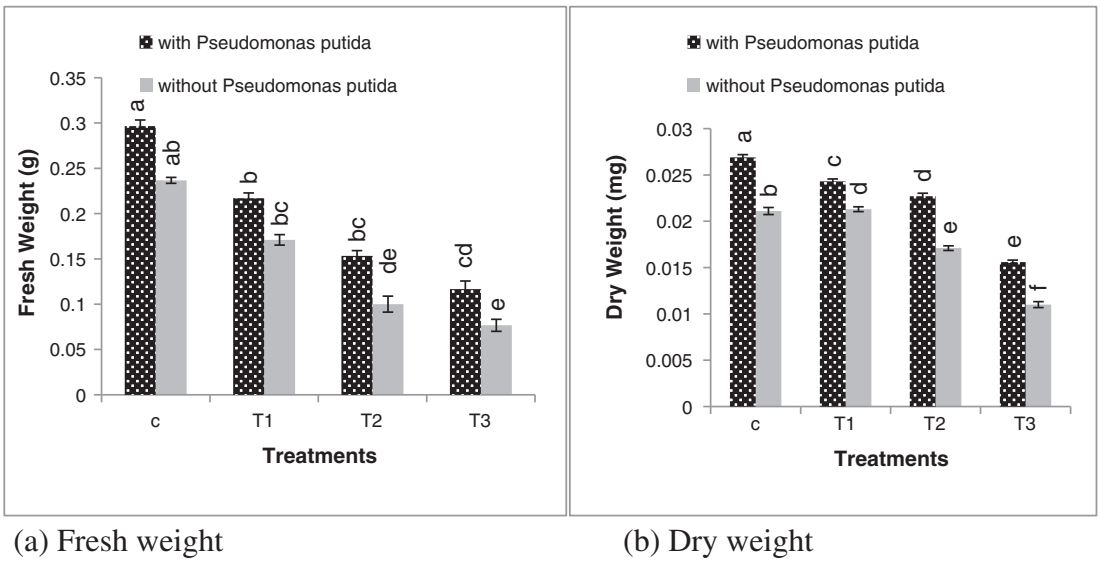
3.3. Effect of P. putida on plant growth and biomass

The present results clearly indicated the significant effect ($p < 0.05$) on the growth of *P. putida* inoculated plants under the Cr stress (at three different levels) in comparison to un-inoculated plants. *E. sativa* root and shoot length was observed to be decreased up to 42% and 39% respectively (Fig. 1 a,b), as Cr contamination increased. Similarly, fresh and dry biomass decreased up to 51% and 43%, respectively (Fig. 2 a, b). However, the inoculation of PGPR increased 14%, 18%, 24% and 33% of the root length and 10% 19%, 28% and 42% of shoot length in PGPR inoculated plants than those of un-inoculated plants from control, low, medium and high levels of Cr contamination (Fig. 1). While, PGPR inoculated plants of *E. sativa* showed 17% 11%, 32% and 49% increase in the fresh weight and 8% 16%, 35% and 53% of dry weight than those of non-inoculated plants grown in the control, low, medium and high levels of Cr contaminated soil (Fig. 1). PGPR improves the plant growth under Cr stress by increasing shoot and root length of inculcated plant (Rajkumar and Freitas, 2008). Plant ability to cope with a wide range of environmental stresses has been effected by mutualistic association crafted by PGPRs including free living rizospheric bacteria (Burd et al., 2000; Mantelin and Touraine, 2004). PGPRs also produce indole acetic acid (IAA) which increase the plant biomass under toxic metal stress conditions and increase the root and shoot length of the plant (Amico et al., 2008; Mia et al., 2010). Dary et al. (2010) reported that under metal stress condition *Lupinus luteus* plants showed much better growth when they were inoculated with *Bradyrhizobium* sp. as compared to those which were non-inoculated. Under stress conditions production of excessive ethylene is general phenomena in plants. Ethylene plays a key role in the growth of different plants however, it inhibits the plant growth if present in excessive quantities (Grichko and Glick, 2001).PGPRs control the quantity of ethylene by consuming 1-aminocyclopropane-1-carboxylate and increase the plant growth and total biomass production (Grichko and Glick, 2001; Mayak et al., 2004). According to Baharlouei et al. (2011), in canola and barley PGPRs strengthened the plant growth by enhancing the root and shoot biomass under heavy metal stress. Similarly in *Brassica napus* plants PGPR promoted the production of root and shoot biomass under stress conditions (Belimov et al., 2001). Moreover, by decreasing ethylene level and production of different phyto- hormones (i.e. Gibberellins, IAA, Auxin)



(a) Root length (b) Shoot length

Fig. 1. Root and Shoot length of *Eruca sativa* grown in Cr polluted soil as a function of PGPR inoculation. Each value is mean of four data at $p \leq 0.05$ level and the bars in the columns represent the standard error. C = control; T1 = 150 μg/g; T2 = 250 μg/g; T3 = 500 μg/g.



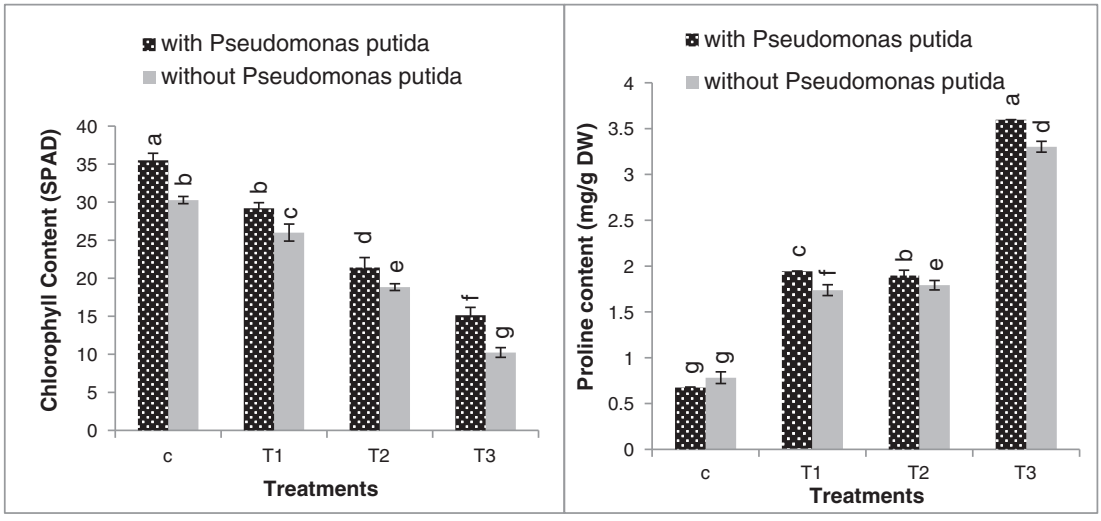
(a) Fresh weight (b) Dry weight

Fig. 2. Fresh and dry weight of *Eruca sativa* grown in Cr polluted soil as a function of PGPR inoculation. Each value is mean of four data at $p \leq 0.05$ level and the bars in the columns represent the standard error. C = control; T1 = 150 μg/g; T2 = 250 μg/g; T3 = 500 μg/g.

PGPR promotes the biomass production and increase the crop yield (Rajkumar and Freitas, 2008; Baharlouei et al., 2011). ACC deaminase hydrolyze aminocyclopropane-1-carboxylate (ACC) that is used as a sole source of Nitrogen by PGPRs, it increases the plant growth and biomass production (Belimov et al., 2001: Amico et al., 2008). Moreover, PGPRs increase the fresh and dry biomass and cope with toxic metals by diminishing their adverse effects on plants (Dary et al., 2010). Burd et al. (2000) studied the *Kluyvera ascorbata* a metal resistant bacterial strain, results suggest that it promotes the plant growth in Ni and other heavy metal contaminated water-soil system. PGPR inhibits the heavy metal toxicity by producing different type of hormones which convert these metals into less toxic form and ultimately increase the plant growth.

3.4. Effect of P. putida on Chlorophyll and proline contents

Chlorophyll and proline contents of *E. sativa* plants were significantly influenced by Cr toxicity. Chlorophyll contents decreased as Cr toxicity increased in *E. sativa* plants by, 21%, 39% and 59% at 150 μg/g, 250 μg/g and 500 μg/g respectively as compared to un-inoculated control (Fig. 3, a). However, PGPR inoculation increased chlorophyll contents by 13%, 24%, 37% and 39% by inoculated control, 150 μg/g, 250 μg/g and 500 μg/g as compared to un-inoculated plants of *E. sativa*. Contrary, Proline contents increased up to 44% with increasing in Cr toxicity as compared to un-inoculated control. However, 17%, 22%, 29% and 44% proline contents increased in inoculated plants as compared to un-inoculated plants (Fig. 3, b). Several studies reported in literature suggest that chlorophyll contents in wheat plants decreased under metals stress conditions, similarly *Typha latifolia* grown in sewage sludge flooded soils also exhibited decreased in chlorophyll contents by heavy metal stress (Manios et al., 2003; Gajewska et al., 2006). However, PGPR inoculation overturned the results by supporting the defense mechanism due to the production of different enzymes which increased the chlorophyll contents (Amico et al., 2008). Proline plays a significant role in proteins chemistry, scavenge free radicals, buffer cellular redox potential and provide membrane strength. Under any environmental



(a) Chlorophyll (b) Proline contents

Fig. 3. Chlorophyll and proline contents of *Eruca sativa* grown in Cr polluted soil as a function of PGPR inoculation. Each value is mean of four data at $p \leq 0.05$ level and the bars in the columns represent the standard error. C = control; T1 = 150 μg/g; T2 = 250 μg/g; T3 = 500 μg/g.

stress proline plays an important role in regulating the osmotic potential and stabilize the structure of many organelles at cellular level (Abdul et al., 2007). Accumulation of proline in plants also serves as environmental stress indicator (Ahmad and Jhon, 2005). A study revealed that in different wheat varieties when seeds were inoculated with PGPRs, proline contents significantly increased under Pb stress (Janmohammadi et al., 2013). Moreover, due to inhibition of degradation steps or de novo synthesis, excessive proline level exist under heavy metal stress condition. Under metal stress conditions, in the stems of different plants species Viz*; Triticum aestivum*, *Barassica juncia* and *Vigna radiate* enhancement in proline contents have been reported (Dhir et al., 2004).

3.5. Effect of P. putida on Cr uptake

Inoculated and un-inoculated plants showed significant difference regarding Cr uptake in *E. sativa* plants. Cr accumulation was more in *P. putida* inoculated plants by 27%, 34% and 38% as compared to un-inoculated plants of *E. sativa* (Fig. 4). Inoculation of rhizobacteria altered the soil chemistry and increased the solubility and bioavailability of different metals for accumulation in different plant parts (Burd et al., 2000; Lasat, 2002). Our results are in agreement of Ma et al. (2011) who observed that *Pseudomonas* sp. A3R3, a plant growth promoting bacteria improved metal uptake in *Alyssum serpyllifolium* under Ni stress as compared to the control, similarly in *Solanum nigrum* Cd accumulation (root and shoot) increased by addition of plant growth promoting bacteria (Chen et al., 2010). Rhizobacteria aids in nutrient and metal uptake of the plant by decreasing the soil pH (Abou-Shanab et al., 2006; Zhuang et al., 2007). The *P. putida* produce different enzyme which also increase the metal uptake (Ma et al., 2011). Endophytes accelerate or degrade the heavy metals present in the soil and hence play a crucial role to combat against heavy metal contamination in plants (Chen et al., 2010; Zhang et al., 2011). In metal contaminated areas wild plants are adversely affected by phyto-toxicity as they have great potential towards metal accumulation. PGPRs inoculation govern the whole process, it produce various metal degrading enzymes, organic acid, iron chelators and sidrosphore which inhibit the phyto-toxic effect for wide range of pollutants (Saravanan et al., 2007; Yousaf et al., 2010). Present study demonstrate that for the growth of *E. sativa* under Cr stress, PGPR having ACCD activity was essential and it was depends on the bacterial strain and its PGP traits. Connolly et al. (2003) reported that iron (Fe^{3+}) present under aerobic conditions forms insoluble hydroxides which are not readily available to plants under low iron condition. Sidrosphore acts as a solubilizing agent, it forms stable complexes with different heavy metals i.e. Cd, Pb etc. and increases the uptake of different minerals (Madhaiyan et al., 2007; Rajkumar et al., 2010). Results suggest that by addition of PGPR bacteria, Cr can be reduce in *Methylobacterium oryzae* and *Burkholderia* sp. of rice. Studies suggest that metal resistant plant growth promoting bacteria can increase metal uptake and accumulation in different plant parts.

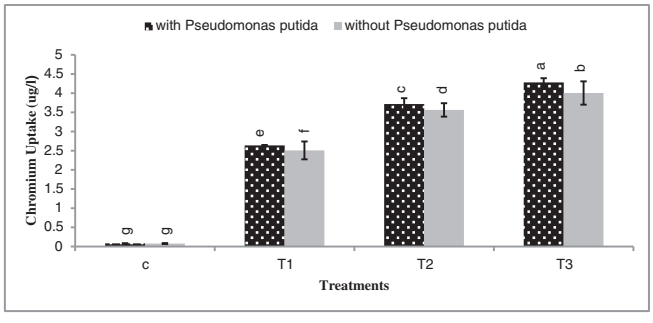


Fig. 4. Chromium uptake by *Eruca sativa* grown in Cr polluted soil as a function of PGPR inoculation. Each value is mean of four data at $p \leq 0.05$ level and the bars in the columns represent the standard error. C = control; T1 = 150 μg/g; T2 = 250 μg/g; T3 = 500 μg/g.

4. Conclusions

The finding showed that PGPR inoculation with plants (*P. putida*) increased plant growth and metal uptake, however microbe-assisted phytoremediation enhance the Cr accumulation and reclaim the Cr toxic soils. Our study demonstrated that the growth promoting effect of PGPR having ACCD activity was essential for the growth of *E. sativa* in the presence of toxic Cr concentrations and it was dependent on the bacterial strain and its PGP traits. Pot experiments demonstrated that the application of PGPR protects plants against the toxic effects of Cr and effectively promote the growth of *E. sativa* and thus increase phytoremediation efficiency. All the traits of PGPR might not be expressed at a given point in time. While ACCD activity may be responsible for better root growth in the initial stages of plant growth, siderophores and IAA production may facilitate the mobilization of nutrients, hormonal balance and, thus, plant growth. Extensive research in the areas of colonization capability, the role of rhizobacteria and plant roots in the uptake of metals and their mode of metal translocation, is required to elucidate the mechanisms of PGPR protection against toxic elements in soil to achieve the stabilization, revegetation and remediation of metal-polluted soils.

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