EnvironmentAsia
The international journal published by the Thai Society of Higher Education Institutes on Environment

Available online at www.tshe.org/EA
EnvironmentAsia 7(1) (2014) 89-94

Enhanced Cadmium (Cd) Phytoextraction from Contaminated Soil using Cd-Resistant Bacterium

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Abstract

A cadmium (Cd)-resistant bacterium, *Micrococcus* sp. MU1, is able to produce indole-3-acetic acid and promotes root elongation and plant growth. The potential of this bacterium on enhancement of Cd uptake and bioaccumulation of Cd in *Helianthus annuus* L. planted in Cd-contaminated soil was evaluated in greenhouse condition. The results showed that *Micrococcus* sp. MU1promoted the growth of *H. annuus* L. by increasing the root length, stem height, dry biomass, root to shoot ratio and also significantly increased Cd accumulation in the root and above-ground tissues of *H. annuus* L. compared to uninoculated control. Re-inoculation with *Micrococcus* sp. MU1in contaminated soil helped in promoting plant growth and Cd phytoextraction throughout the cultivation period. In addition, phytoextraction coefficient and translocation factor (TF) of *H. annuus* L. inoculated with *Micrococcus* sp. MU1were higher than that of uninoculated control and TF continuously increased with time. Our results suggested that *Micrococcus* sp. MU1 has an ability to enhance plant growth and Cd uptake in *H. annuus* L. Synergistic interaction between *Micrococcus* sp. MU1 and *H. annuus* L. could be further applied for Cd phytoextraction in polluted areas.

Keywords: cadmium; soil; *Micrococcus* sp.; phytoextraction; *Helianthus annuus* L.

1. Introduction

Cadmium (Cd) is one of toxic heavy metals that cause adversely effects on both human health and the functioning of ecosystems (Perronnet *et al.*, 2000). It can transfer from the soil to plants via plant root system and accumulate into the plant tissues. Subsequently, it enters to animal and human body via the food chain (Rai *et al*., 1981). The removal of Cd from the soil is an urgent need to be solved the soil pollution in order to avoid its transfer in the food chain (Grytsyuk *et al*., 2006). Phytoextraction is use of live plants to uptake and accumulation of heavy metals in the different parts of the plant tissues (Branquinho *et al*., 2007). However, the treatment of Cd using live plant alone has some limitations e.g. low plant growth rate due to Cd toxicity, slow rate of Cd uptake by plants due to low metal bioavailability in soil (Lebeau *et al*., 2008; Prapagdee *et al*., 2013).

Plant growth-promoting rhizobacteria (PGPR) are plant-associated bacteria that able to promote plant growth by colonizing in the plant root (Glick, 2010). PGPR can produce phytohormones, particularly indole-3 acetic acid (IAA). IAA is an important plant growth hormone to stimulate shoot and root elongation, lateral root initiation, plant growth and development (Patten and Glick, 1996). Bioaugmentation with bacteria-assisted heavy metal phytoextraction is a promising method for clean-up of heavy metal-contaminated soils (Lebeau *et al*., 2008). The synergistic interactions between plant and rhizobacteria to promote plants growth and Cd uptake resulting in the better performance of Cd phytoextraction are reported by several investigators (Zhuang *et al*., 2007; He *et al*., 2009; Prapagdee *et al*., 2013).

Sunflower (*Helianthus annuus* L.) is widely used for phytoremediation of several heavy metals in soil e.g. Cd, chromium, nickel (Turgut *et al*., 2004; Padmavathiamma and Li, 2007). The study of Andaleeb *et al*. (2008) reported that chromium accumulates in sunflower seeds less than in the roots and shoots. Oil extracted from sunflower seeds could be safely used for biodiesel. No detectable amount of Cd, copper and lead has been observed in oils extracted from aromatic crops planted in heavy contaminated soils (Zheljazkov *et al*., 2006). However, only one time inoculation with PGPR in Cd contaminated soil cannot prolong the efficiency of Cd phytoextraction for long time (Prapagdee *et al*., 2013). Therefore, this study elucidates the combined approach using *H. annuus* L. and continuous inoculation with Cd-resistant PGPR, *Micrococcus* sp. MU1, to promote Cd phytoextraction in Cd-polluted soil.

2. Materials and Methods

2.1. Bacterial strain and bacterial inoculum preparation

The strain of Cd-resistant bacterium, *Micrococcus* sp. MU1, was isolates from plant root by Chanprasert *et al.* (2011). The minimum inhibitory concentrations of Cd for *Micrococcus* sp. was 1000 mM and it produced high levels of IAA up to 402 ± 13 mg/L (Prapagdee *et al*., 2013). For bacterial inoculum preparation, overnight cells of *Micrococcus* sp. MU1was inoculated in Luria-Bertani (LB) broth supplemented with 0.5 mg/mL L-tryptophan and incubated at 28°C with continuous shaking at 150 rpm for 36 hr. Method for inoculum preparation was previously described by Prapagdee *et al*. (2013). Cell turbidity was measured using spectrophotometer at the wavelength of 600 nm (OD_{600}) and adjusted to an OD_{600} of 0.2 for use as cell inoculum in the pot experiments.

2.2. Preparation of H. annuus L. seedlings

H. annuus seedlings were planted in uncontaminated garden soil and grown for 2 weeks (20-cm-height). The seedlings were gently removed and transplanted to Cd-contaminatedsoil in 25-cm-diameter of plastic pots.

2.3. Soil collection and preparation

Contaminated agricultural soil was collected from Cd-polluted area at Mae Sot district, Tak province, Northern Thailand. Soil was air-dried, ground, and passed through a 2-mm sieve. Contaminated soil sample was analyzed for Cd amount with flame atomic adsorption spectrophotometer (FAAS) (Varian spectra model AA240FS, USA).

2.4. Experimental design in greenhouse condition

Seedlings of *H. annuus* L. were directly transplanted in plastic pots containing 3 kg of Cd-contaminated soil and randomly placed in a greenhouse for 9 weeks. This experiment was divided into 2 treatments as follows: (i) 2-week-old *H. annuus* L. with no bacteria added (the uninoculated control) and (ii) 2-week-old *H. annuus* L. inoculated with *Micrococcus* sp. MU1. Each treatment was performed at least in triplicate. All pots were daily watered in the morning. Fresh culture of *Micrococcus* sp. MU1 was continuously inoculated in soil in bacterial treatment every 3-week-intervals to ensure the presence of viable bacterial cells in soil.

2.5. Plant and soil analysis

Plant and soil samples were collected at 3, 6 and 9 weeks, respectively. Each harvested plant was thoroughly washed with tap water and rinsed twice with deionized water. Plants were measured for stem height and root length and divided into the shoot (leaf and stem) and root parts. Each part of plant was oven-dried and weighed. Plant and soil samples were acid-digested and Cd concentration was analyzed with FAAS.

2.6. Data and statistical analysis

To compare plant growth between 2 treatments, the ratio of dry weight roots to dry weight shoots was calculated (Chiu *et al*., 2006). Cd concentration in leftover soil and plant samples was analyzed to find out phytoextraction coefficient and translocation factor (TF) by Kumar *et al*. (1995) and Mattina *et al*. (2003). Data of plant growth, Cd concentration in each plant part, phytoextraction coefficient and TF were calculated ($\overline{x}$) along with standard error (SE). To compare the means of different treatments, data of plant growth and Cd contents in plants from both treatments were statistically analyzed using one-way analysis of variance at 95% confidence intervals followed by a Duncan's multiple range test at $p < 0.05$.

3. Results and Discussion

3.1. Plant growth promotion of in Cd-contaminated soil by Cd-resistant bacterium

Our previous study showed that *Micrococcus* sp. MU1, an IAA-producing rhizobacteria or PGPR, was able to stimulate root elongation and the growth of *H. annuus* L. under toxic Cd condition but plant growth of root and biomass by this bacterium was decreased with time (Prapagdee *et al*., 2013). Therefore, re-inoculation with *Micrococcus* sp. MU1 in Cd-polluted soil was performed. Cd concentration in the tested soil was 53.8 mg/kg. The results exhibited that the root length and stem height of *H. annuus* L. inoculated with *Micrococcus* sp. MU1 at the 9 week after transplant were significantly higher compared to uninoculated "control at $p < 0.05$ (Table 1). After 9th week of cultivation, inoculation of *Micrococcus* sp. MU1 increased the root length and stem height of *H. annuus* L. by 1.2 and 10.2 cm, respectively compared to the uninoculated control. Compared to uninoculated control, *Micrococcus* sp. MU1 significant increased dry biomass weight of whole plant and the root to shoot ratio after transplant for 6 and 9 weeks (Table 2). The dry biomass weight

Table 1. Root length and stem height of *H. annuus* L. after growth in Cd-contaminated soil

Treatment	Root length (cm)			Stem height (cm)		
	Week 3	Week 6	Week 9	Week 3	Week 6	Week 9
No bacteria inoculation	0.8±0.4a	4.5±0.4a	5.2±0.3a	17.4±0.8a	26.2±0.9a	31.9±0.5a
Micrococcus sp. MU1	0.9±0.3a	4.5±0.2a	6.4±0.3b	16.3±3.9a	38.8±1.9b	42.1±3.9b

The means and the S.E. ($n = 3$) followed by the same letter within a column were not significantly different ($p < 0.05$) according to Duncan's multiple range test.

Table 2. Plant dry weight and root to shoot ratio of *H. annuus* L. after growth in Cd-contaminated soil

Treatment	Dry weight of whole plant (g/plant)			Root to shoot ratio		
	Week 3	Week 6	Week 9	Week 3	Week 6	Week 9
No bacteria inoculation	0.54±0.16a	0.91±0.11a	1.28±0.03a	0.06±0.01a	0.01±0.00a	0.06±0.00a
Micrococcus sp. MU1	0.67±0.14a	1.39±0.09b	8.47±0.19b	0.08±0.05a	0.35±0.17b	0.12±0.01b

The means and the S.E. ($n = 3$) followed by the same letter within a column were not significantly different ($p < 0.05$) according to Duncan's multiple range test.

of *H. annuus* L. inoculated with *Micrococcus* sp. MU1 at 6 and 9 weeks after transplant increased by 0.48 and 7.19 g, respectively compared to uninoculated control. The high root to shoot ratio indicated that *Micrococcus* sp. MU1 promoted the root proliferative of *H. annuus* L. planted in Cd-contaminated soil.

During plant uptake of Cd from soil, Cd inhibited plant root and shoot lengths and interferes with uptake and transport of several metals (Benavides *et al.*, 2005). Our findings suggested that *Micrococcus* sp. MU1 was capable to increase the plant growth parameters by a continuous supply of IAA to *H. annuus* L. IAA positively influences root growth and plant development, thereby enhancing nutrient uptake (Ahmad *et al*., 2008). The shoot tissues are more sensitive to heavy metal toxicity than root tissues. Exposure to heavy metal, the biomass of shoot tissue was more decreased than that of root (Kahle, 1993; Chiu *et al*., 2006). Several investigators have reported that heavy metal-resistant rhizobacteria promote plant growth cultivated in heavy-metal contaminated soils. *Burkholderia* sp. J62, an IAA-producing Cd and Pb-resistant bacterium increased the shoot and root dry weights of corn (*Zea mays* L.) and tomato (*Lycopersicon esculentum*) plants (Jiang *et al*., 2008). He *et al*. (2009) reported that *Pseudomonas* sp. RJ10 and *Bacillus* sp. RJ16, Cd-resistant bacteria, were able to produce IAA and increased the root and shoot tissue dry weights of hyperaccumulator tom to planted in heavy metal-contaminated soil compared to inoculation with dead bacterial cells.

3.2. Cd-resistant bacterium assists the Cd uptake and bioaccumulation in H. annuus L.

The increases in plant biomass by *Micrococcus* sp. MU1 prompted us to investigate the ability of *Micrococcus* sp. MU1 to enhance Cd phytoextraction by *H. annuus* L. planted in Cd-contaminated soil. The results showed that Cd content in the roots of *H. annuus* L. inoculated with *Micrococcus* sp. MU1 at 9 weeks after transplant was higher than that of the uninoculated control (Fig. 1). *Micrococcus* sp. MU1 enhanced Cd accumulation in the above-ground tissues of *H. annuus* L.The highest Cd accumulation in *H. annuus* L. was found in the above-ground tissues at 3 week after transplant by 20.11±2.48 µg/g dry biomass. These results suggested that *H. annuus* L. has strongly capable to accumulate highly Cd concentration and rapidly translocate to the above-ground plant tissues.

Cd accumulation in different parts of plants in descending order shows as follows: root > stem > leaf > fruit > seed (Benavides *et al*., 2005). Generally, metal hyperaccumulators are plants that can accumulate high metal concentrations in their above-ground tissues without visible symptoms of metal toxicity (Padmavathiamma and Li, 2007). The application of *Micrococcus* sp. MU1 significantly increased Cd uptake and accumulation in whole plant (Fig. 2). In comparison to the uninoculated control, Cd accumulation in whole plant of *H. annuus* L. inoculated with *Micrococcus* sp. MU1 at 3, 6 and 9 weeks after transplant increased by 2.4-, 6.6- and 2.4-fold, respectively. These findings

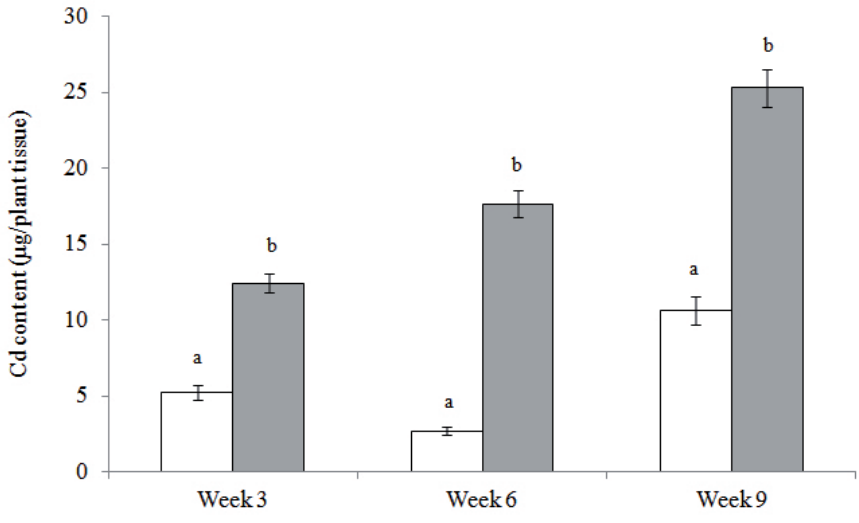

Figure 2. Cd contents in whole plant of *H. annuus* L. planted in Cd-contaminated soil inoculated with (▭) *Micrococcus* sp. MU1 and with no bacterial inoculation (□). The error bars are the S.E. ($n = 3$), and the small letter above the bar graph denotes a significant difference ($p < 0.05$).

indicated that continuous inoculation with *Micrococcus* sp. MU1 can help to prolong the ability of *Micrococcus* sp. MU1 on the enhancement of Cd uptake and accumulation in *H. annuus* L. throughout the cultivation period.

Pseudomonas sp. RJ10 and *Bacillus* sp. RJ16, Cd-resistant bacteria, increased Cd accumulation compared to the uninoculated control in rape (*Brassica napus*) planted in Cd-spiked soil at concentration 100 mg/kg (Sheng and Xia, 2006). He *et al*. (2009) reported that Cd concentrations in the above-ground tissues of tomato amended with either *Pseudomonas* sp. RJ10 or *Bacillus* sp. RJ16 were increased varying from 92% to 113% compared to the uninoculation control. Cd uptake in the above-ground tissues of tomato was increased by 9.3- to 10.0-fold compared to that in tomato roots in bacterial-inoculated soil (He *et al*., 2009). In addition, Cd contents in soil planted with *H. annuus* L. in the presence and absence of *Micrococcus* sp. MU1 were significantly reduced after plantation for 9 weeks.

3.3. Potential of Cd-resistant bacterium for its phytoextraction and translocation in H. annuus L.

In general, plant performance on heavy metal removal and translocation of heavy metals from the roots to the above-ground tissues involved phytoextraction coefficient and translocation factor (TF). Plants with the high phytoextraction coefficient and TF have the potential for metal phytoextraction (Yoon *et al*., 2006; Nouri *et al*., 2011). Our results revealed that phytoextraction coefficient and TF of *H. annuus* L. inoculated with *Micrococcus* sp. MU1 in all collected periods were higher than those of the uninoculated control (Fig. 3). Although, *H. annuus* L. is not the best Cd hyperaccumulator due to low phytoextraction coefficient but the oil extracted from *H. annuus* L. seeds without Cd contamination offers economical benefits for reclamation of polluted areas. Interestingly, TFs of *H. annuus* L. inoculated with Micrococcus sp. MU1 in all collected periods were higher than those of the uninoculated control. Similar to the phytoextraction coefficient, the highest phytoextraction coefficient (0.5) and TF (1.7) were found at 3 week after transplant *H. annuus* L. in contaminated soil with bacterial inoculation.

Cd around the plant root is able to translocate into the above-ground plant tissues; however, Cd is generally more persist in the root than translocate to the shoots (Benavides *et al*., 2005). *Micrococcus* sp. MU1 increased Cd translocation from the roots to the shoots. These results were supported by the study of Prapagdee *et al*. (2013) which reported that *Micrococcus* sp. MU1 was able to increase Cd solubility. Increasing metal solubility or bioavailability in soil promotes metal uptake and translocation from plant roots to its shoots (Chiu *et al*., 2006). Our findings suggested that re-inoculation of contaminated soil with *Micrococcus* sp. MU1 can lead to higher plant uptake and translocation of Cd from soil to above-ground plant tissue.

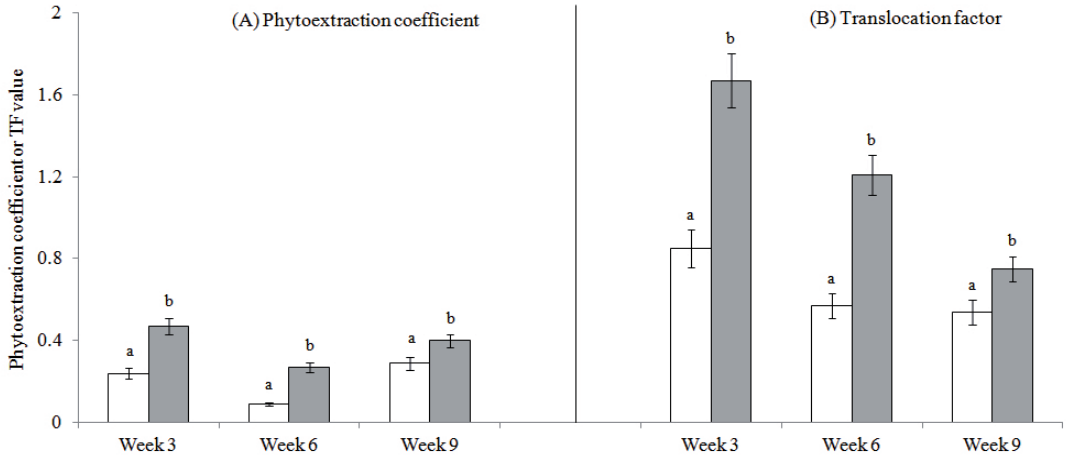

Figure 3. (A) Phytoextraction coefficient and (B) translocation factor of Cd in *H. annuus* L. planted in Cd-contaminated soil inoculated with (▭) *Micrococcus* sp. MU1 and with no bacterial inoculation (▭). The error bars are the S.E. ($n = 3$), and the small letter above the bar graph denotes a significant difference ($p < 0.05$).

4. Conclusions

Micrococcus sp. MU1, a Cd-resistant PGPR, promoted the plant growth and Cd accumulation in the roots and above-ground tissues of *H. annuus* L. It also increased phytoextraction coefficient and translocation of Cd from the roots to the above-ground tissues. Repeated soil inoculation with the *Micrococcus* sp. MU1 provided continuous enhancement of Cd phytoextraction efficiency. Our findings suggested that a combined approach using *Micrococcus* sp. MU1 and *H. annuus* L. for Cd phytoextraction is an alternative strategy to reclaim Cd-polluted area.

Acknowledgements

This research was supported by Laboratory of Environmental Biotechnology, Faculty of Environment and Resource Studies, Mahidol University.

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Received 19 November 2013
Accepted 9 December 2013

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