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ARTICLE

Effect of hemoglobin on the growth and Cd accumulation of pea plants (*Pisum sativum* L.)

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Abstract This study was set to investigate the effect of the presence of hemoglobin (Hb) in cadmium (Cd)-contaminated soil on phytotoxicity and Cd accumulation. The effect of Hb on the Cd accumulation by *Pisum sativum* L. (pea) and seed germination and growth was studied using pot tests with the artificially Cd-contaminated soil. The results show that the externally applied Hb to Cd-contaminated soil samples did not promote Cd accumulation by *P. sativum*. However, the Fe accumulation was greater in the presence of Hb. The seed germination was not affected, but the adverse effects on the plant growth increased with increasing Hb/Cd molar ratio from 0 to 0.015. This can be attributed to toxic effects of the Fe added with the Hb application. The results suggest that the presence of Hb may have harmful effects on pea plants used in phytoremediation of Cd-contaminated soil due to toxic effects imposed by Fe.

Keywords Cadmium · Hemoglobin · Phytoremediation · *Pisum sativum*

Introduction

Organic contaminants such as total petroleum hydrocarbons (TPHs) and polycyclic aromatic hydrocarbons (PAHs) and heavy metals such as lead (Pb) and cadmium (Cd) are often found together in contaminated sites. Biological processes such as bioremediation have been widely applied to degrade organic contaminants; however, the same treatment may not be efficient for heavy metal removal as they are not biodegraded. Conventionally, techniques such as soil washing, soil flushing, and solidification and stabilization have been used for remediation of heavy metal-contaminated soils [1]. However, physical and/or chemical techniques often involve high cost, irreversible changes in soil properties, and disturbance of native soil microflora [2, 3]. When biological processes are preferred for heavy metal removal, phytoremediation can be used to remove heavy metals from heavy metal-contaminated soils [4]. But heavy metals can impose harmful effects on plants upon exposure [5–9].

Recently, soils with various organic contaminants have been treated using hemoglobin (Hb)-catalyzed biocatalytic reactions [10, 11]. The Hb applied for the biocatalytic reaction is likely to remain in the contaminated site after removal of organic contaminants. Since Hb has a Fe-containing heme structure, Hb can be a source of iron (Fe) to the plants under heavy metal stress. Thus, the Hb remaining in the contaminated sites after biocatalytic reaction might reduce toxic effects imposed by heavy metals on plants (i.e., enhanced resistance to heavy metal stress). This, in turn, may help promoting phytoremediation of heavy metals from the contaminated sites. However, the effect of externally applied Hb on plants has not been studied.

Previous studies used transgenic plants to manipulate plant resistance to heavy metals, heavy metal uptake and accumulation, and translocation in order to enhance phytoremediation efficiency [12–15]. In particular, various transgenic plants with Hb genes have been studied to investigate the role of Hb in plants under stresses, and these

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studies showed that overexpression of plant Hb enhanced tolerance to stresses due to hypoxia and heavy metals, and enhanced resistance to diseases [16–18]. When plants are under stress due to heavy metals such as Pb, reactive oxygen or nitrogen species such as nitric oxide (NO) are produced triggering responses at gene expression level for scavenging of reactive species [19]. Although there are different observations on the relationship between NO and heavy metal toxicity, in particular, Cd toxicity, overexpression of plant Hb gene resulted in a reduced NO level [20]. On the other hand, the NO produced may promote heavy metal uptake and this can contribute to toxic effect by heavy metals [16]. However, Hb scavenges NO induced by environmental stresses in plants [21]. For example, tobacco expressing the *NtHb1* gene encoding Hb alleviated the Cd-induced NO level, and exhibited higher Cd tolerance and lower Cd accumulation [22]. When transgenic plants are used in phytoremediation, there are some possible risks involved such as uncontrolled spread of transgenic plants and risk of ingestion by wildlife [14].

Some studies supplied nutrients in order to alleviate toxicity of heavy metals [23]. For example, Fe can be used to alleviate Cd toxicity by limiting Cd uptake and enhancing photosynthesis [24]. Thus, Fe supplements may enhance the plant resistant to heavy metal stress, which, in turn, affect the phytoremediation efficiency [24]. Therefore, this study is set to investigate the effect of the presence of Hb (e.g., residual Hb after Hb-catalyzed biocatalytic reactions for organic contaminant removal) on plants. Pea is one of common crops that has showed high potential in phytoremediation [25]. Also, legume-rhizobia symbiosis-based phytoremediation for Cd removal is getting more attention [4]. Therefore, the effects of the presence of heme protein-containing Hb in Cd-contaminated soils on the growth of pea plants and the Cd accumulation by pea plants were studied.

Materials and methods

Soil preparation

Soil samples were prepared by air-drying and sieving through a 2-mm sieve. The soil texture was loamy sand with the organic matter content, pH, and water holding capacity of $10 \pm 0.028\%$, 6.5, and $17 \pm 2.4\%$, respectively. The background concentrations of Pb, Cd, As, Cu, and Zn were 35 ± 0.28, < 0.0048 (i.e., not detected), 5.3 ± 0.064, 37 ± 0.85, and 300 ± 5.2 mg kg^{-1}, respectively. The soil sample was artificially contaminated with a Cd solution prepared from $CdSO_4$ (99% Junsei Chemical Co., Tokyo, Japan) to have the soil Cd concentration of 6.0 ± 0.61 mg kg^{-1}. The Cd concentration of the soil sample was chosen based on the worrisome levels of the residential area in the Korean Soil Standard.

Pot tests with the Cd-contaminated soil

Phytotoxicity tests are widely carried out to see the effect of contaminants on the seed germination and plant growth [26, 27]. The phytotoxicity tests were carried out following the OECD 208 method, which describes the seedling emergence and seedling growth test methods for terrestrial plants [28]. The effect of Hb in soil on the Cd accumulation by *Pisum sativum* L. (pea) and the seedling growth were studied using pot tests with the Cd-contaminated soil. Pea is a crop plant that has been widely used in the phytotoxicity studies of Cd [29, 30]. For the pot tests, different amounts of Hb were added to the Cd-contaminated soil (300 g) to have the Hb/Cd molar ratios of 0.000, 0.001, 0.009, and 0.015 (i.e., the Hb/Cd mass ratios of 0, 0.8, 5.0, and 8.4). Hb powder was purchased from Shenzhen Taier Biotechnology Co., Ltd. (Shenzhen, China). To compare the Cd accumulation and plant growth in the soil sample without artificial Cd contamination, the same amounts of Hb added to the Cd-contaminated soil at the Hb/Cd molar ratios of 0.000 and 0.015 were added. Each pot contained four seeds of *P. sativum,* and the pot tests were carried out in duplicates for each condition. The pot tests were carried out in a growth chamber at an average temperature of 25 °C with the light condition of 18 h day and 6 h night. The soil moisture was maintained at 70% of water holding capacity. The seed germination was checked after 10 d, when the germination in the controls (i.e., without Cd contamination and Hb application) reached 50%, to see if the seed germination is affected by the Hb/Cd molar ratios. Also, the seedling growth was compared by measuring the shoot length after 10 (i.e., when the germination was compared) and 28 day. The seedlings were taken out of the pots carefully to prevent any losses in roots, and the whole plants were used for Cd analysis.

Analysis of heavy metals

The collected pea plants were dried at 80 °C for 90 min, and the heavy metals were extracted from the dried pea plants by following the EPA 3052 methods [31]. The extracts were analyzed using inductively coupled plasma–optical emission spectroscopy (ICP–OES). The method detection limits of ICP–OES for Fe and Cd were 0.0033 and 0.0048 mg kg^{-1}, respectively. All statistical analyses (i.e., *t* test and one-way ANOVA, and Tukey's test for the post hoc test, Spearman's correlation analysis) were performed using SPSS (v 21).

Results and discussion

Effect of Hb on seed germination

The Cd contamination and Hb application did not have a significant effect on the seed germination (p value = 0.634) (Fig. 1). In the Cd-contaminated soils, the average seed germination rates at the studied Hb/Cd molar ratios ranged from 83 to 96% (Fig. 1). This suggests that the Hb application did not impose toxic effects on the seed germination. In the soil without artificial Cd contamination (referred to as the Cd-control soil, hereafter), the seed germination rates without Hb and with Hb (i.e., the same amount of Hb applied to the Cd-contaminated soil to have the Hb/Cd molar ratio of 0.015) were 72 ± 26 and 100 ± 0%, respectively, and they did not show a statistically significant difference (p value > 0.05). Furthermore, the seed germination in the Cd-control soil samples did not show a statistically significant difference with the seed germination in the Cd-contaminated soil samples (p value > 0.05). This indicates that the Hb application or Cd contamination did not have any statistically significant influence on the seed germination. A previous study observed that the seed germination of pea was not affected when the Cd concentration was less than 0.5 mM [32]. This study used the average soil Cd concentration of 0.05 mM (i.e., 6.0 mg kg^{-1}), and the bioavailable Cd concentration is likely to be lower than 0.05 mM, which is much lower than 0.5 mM. This explains no statistically significant influence of the Cd contamination on the seed germination.

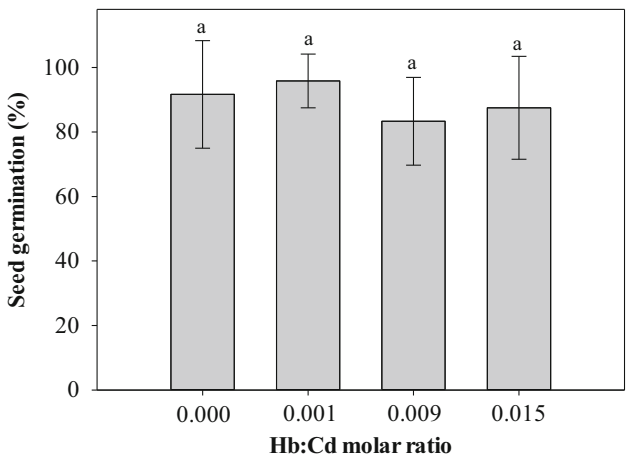

Fig. 1 Effect of hemoglobin-to-cadmium (Cd) molar ratios in the Cd-contaminated soil [6.0 ± 0.61 mg Cd (kg soil)$^{-1}$] on the seed germination of *Pisum sativum*. The different small letters indicate a statistically significant difference (95% confidence level) based on the Tukey's test results

Effect of Hb on accumulation of Cd and Fe

Figures 2A and 1B show the Cd accumulation by *P. sativum* in the Cd-contaminated soils at different Hb/Cd molar ratios. The Cd accumulation by the plant in the Cd-control soils was negligible [0.005 ± 0.001 mg (g dry plant mass)$^{-1}$] compared to the Cd accumulation in the Cd-

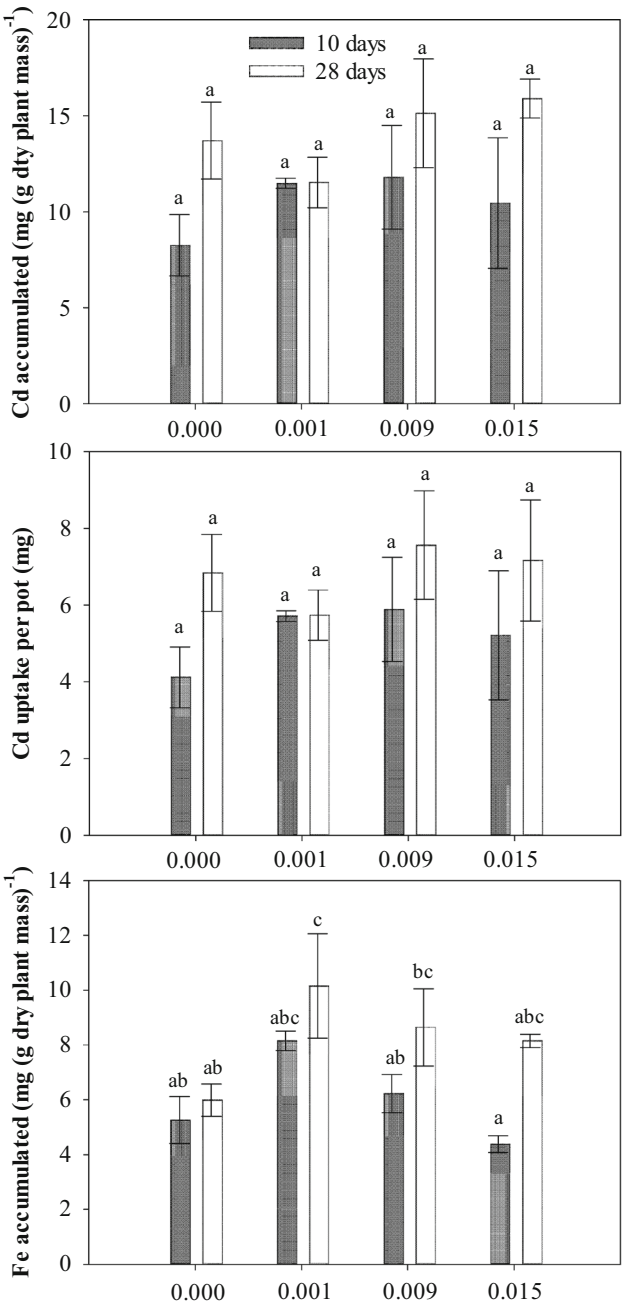

Fig. 2 Effect of hemoglobin-to-cadmium (Cd) molar ratios in the Cd-contaminated soil [6.0 ± 0.61 mg Cd (kg soil)$^{-1}$] on (**A**) the Cd accumulation in unit dry mass of *Pisum sativum*, (**B**) Total mass of the Cd accumulated in one pot, and (**C**) Fe accumulation in unit dry mass of *P. sativum*. The different small letters indicate a statistically significant difference (95% confidence level) based on the Tukey's test results

contaminated soils shown in Fig. 2. The different Hb/Cd molar ratios did not have statistically significant influence on the Cd accumulation per unit mass of the dried plants (p value > 0.05) (Fig. 2A). Similar results were obtained with the Cd uptake per pot (Fig. 2B). This shows that the externally applied Hb to Cd-contaminated soils did not promote Cd accumulation by *P. sativum*.

On the other hand, the Fe accumulation by *P. sativum* in the Cd-contaminated soils was affected by the Hb/Cd molar ratios (Fig. 2C). Since Hb has a Fe-containing heme structure, the applied Hb may supply Fe to the plants. After 10-day exposure to the Cd-contaminated soil, the average Fe accumulation increased with increasing Hb/Cd molar ratio from 0.000 to 0.001 and decreased with further increase in Hb/Cd molar ratio to 0.009 and 0.015 (Fig. 2C). But the Hb/Cd molar ratio did not have any significant influence on the Fe accumulation (p value > 0.05). However, there was a statistically significant monotonic correlation between the Fe accumulation and Hb/Cd molar ratio at Hb/Cd molar ratio $\geq$ 0.001 ($r = -0.956$, p value < 0.01). After 28-day exposure, there was no statistically significant monotonic correlation between the Fe accumulation and Hb/Cd molar ratio at Hb/Cd molar ratio ≥ 0.001 ($r = -0.598$, p value > 0.05). In general, the average Fe accumulation after 28 d was greater in the presence of Hb (i.e., at Hb/Cd molar ratios of 0.01–0.015) than in the absence of Hb (Fig. 2C), and this can be largely attributed to the greater amount of Fe present in the soil by the Hb application. This can be supported by the increased average soil total Fe concentrations from 19,110 mg kg^{-1} at Hb/Cd molar ratio of 0.000–20,859, 21,255, and 21,597 mg kg^{-1} at Hb/Cd molar ratios of 0.001, 0.009, and 0.015, respectively, with the Hb application. After 28 d of the plant growth, the average soil Fe concentrations were decreased by 4.3% in the absence of Hb, but by 12%, on average, in the presence of Hb. This indicates that the Fe added by the Hb application promoted Fe accumulation by the plants.

Effect of Hb on plant growth

Figure 3 shows the growth of *P. sativum* in the Cd-contaminated soils at different Hb/Cd molar ratios. Although Fe is an essential nutrient for plants, an excess amount of Fe can have adverse effects (i.e., iron toxicity) on plant metabolism by interrupting absorption of other nutrients, and by reacting with oxygen producing harmful free radicals [33]. The adverse effects of higher amounts of Fe in the soil can be supported by the statistically lower shoot lengths in the presence of Hb than in the absence of Hb after 10 day (p value < 0.05) (Fig. 3). However, after 28 day, there was no statistically significant influence of Hb on the shoot length (Fig. 3). However, apart from the shoot length, the adverse effects on the physical appearance of the plants

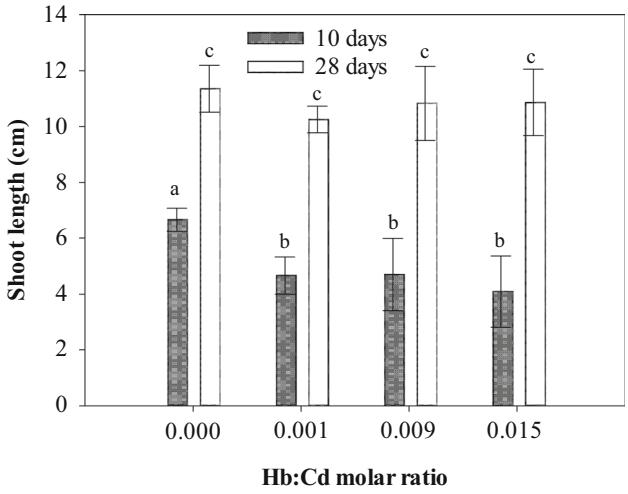

Fig. 3 Effect of hemoglobin-to-cadmium (Cd) molar ratios in the Cd-contaminated soil [6.0 ± 0.61 mg Cd (kg soil)$^{-1}$] on the shoot length of *Pisum sativum*. The different small letters indicate a statistically significant difference (95% confidence level) based on the Tukey's test results

increased with increasing Hb/Cd molar ratios as shown in Fig. 4. The leafy parts of the plants were reduced with increasing Hb/Cd molar ratio. Since the Cd accumulation was not affected by the Hb application (Fig. 2), the adverse effects on the plant growth (Figs. 3, 4) may be attributed to the greater Fe accumulation in the presence of Hb. Similarly, the presence of excess Fe resulted in lower dry weights of pea seedlings suggesting lower growth [34].

Competitive relationship between Cd and Fe

The Fe accumulation was promoted with the Hb application, while the Cd accumulation was not affected (Fig. 2). This also agrees with previous studies that observed competitive uptake relationship between Fe and Cd [24, 35]. Heavy metals such as Cd compete with nutrients such as Ca^{2+} and Fe^{2+} for the transport systems [23]. In particular, Cd interferes with metal uptake and translocation, and this often induces Fe-deficient conditions in the shoot [35, 36]. For example, under Cd stress, mustard plants grown under Fe-fed conditions had higher chlorophyll contents than that grown under Fe-deficient conditions [24]. Although the adverse effect of Cd stress can be reduced by supplying Fe, higher Fe accumulation can exhibit adverse effects on the plants growth.

At Hb/Cd molar ratios of 0.009 and 0.015, there was no statistically significant monotonic correlation between the Cd accumulation and Hb/Cd molar ratio at Hb/Cd molar ratio ≥ 0.001 ($r = 0.717$, p value > 0.05 after 10 day and $r = -0.120$, p value > 0.05 after 28 day) (Fig. 2A). However, after 28 day, the average Cd accumulation seems to be increasing with increasing Hb/Cd molar ratio (Fig. 2A). The average Fe accumulation, on the other hand,

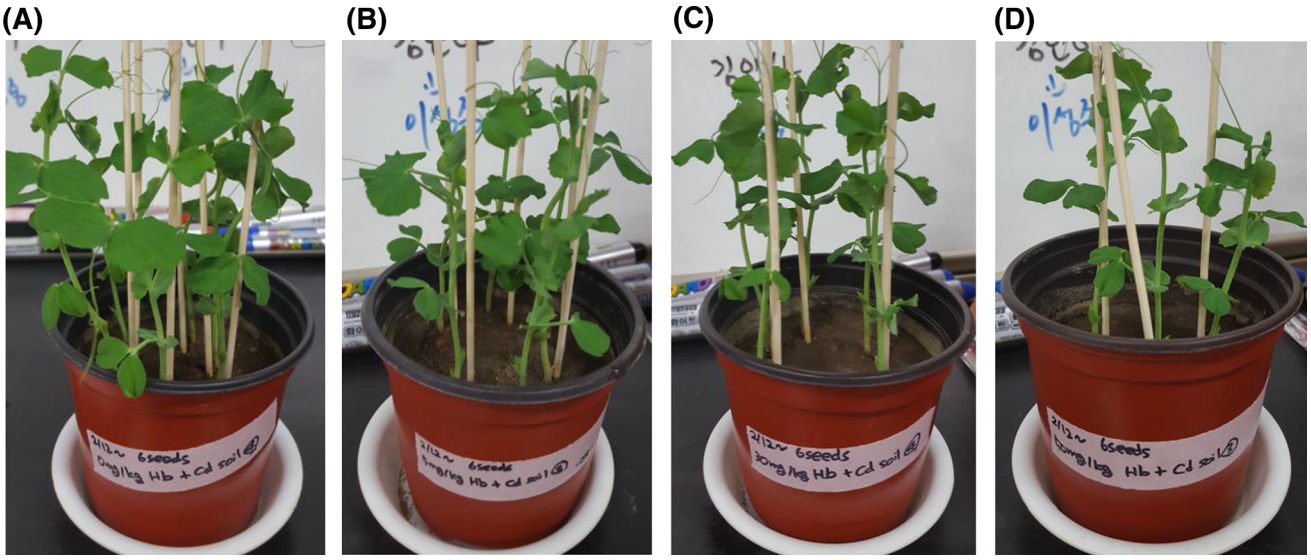

Fig. 4 Growth of *Pisum sativum* in the Cd-contaminated soil [6.0 ± 0.61 mg Cd (kg soil)$^{-1}$] at hemoglobin (Hb)-to-Cd molar ratio of (**A**) 0.000 (No Hb addition), (**B**) 0.001, (**C**) 0.009, and (**D**) 0.015

seems to be decreasing with increasing Hb/Cd molar ratio, although there was no statistically significant correlation (Fig. 2C). These results suggest the competitive relationship between Cd and Fe. Furthermore, in the absence of Hb application, the average Cd accumulation was higher after 28-day exposure, but the average Fe accumulation was similar (Fig. 2). This also supports the competitive relationship between Fe and Cd. Similarly, the higher Cd accumulation rate was observed in the absence of Fe than in the presence of Fe [24].

This study shows that the presence of Hb in the Cd-contaminated soils did not enhance the Cd accumulation by *P. sativum*. The presence of Hb, however, resulted in the adverse effect on the plant growth. This could largely be attributed to iron toxicity due to the Fe supplied by the Hb application. Overall, the presence of Hb is not likely to enhance phytoremediation efficiency of Cd-contaminated soils by pea plants. Thus, it may not be preferred when phytoremediation is to be applied for heavy metal remediation after organic contaminant removal using Hb-based biocatalytic reactions from the sites contaminated with both organic contaminants and heavy metals.

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