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# Effects of heavy metals on seed germination and early seedling growth of *Arabidopsis thaliana*

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**Abstract**

Seed is a developmental stage that is highly protective against external stresses in the plant life cycle. In this study, we analyzed toxicity of essential ($Cu^{2+}$ and $Zn^{2+}$) and non-essential heavy metals ($Hg^{2+}$, $Pb^{2+}$ and $Cd^{2+}$) on seed germination and seedling growth in the model species *Arabidopsis*. Our results show that seedling growth is more sensitive to heavy metals ($Hg^{2+}$, $Pb^{2+}$, $Cu^{2+}$ and $Zn^{2+}$) in comparison to seed germination, while $Cd^{2+}$ is the exception that inhibited both of these processes at similar concentrations. To examine if toxicity of heavy metals is altered developmentally during germination, we incubated seeds with $Hg^{2+}$ or $Cd^{2+}$ only for a restricted period during germination. $Hg^{2+}$ displayed relatively strong toxicity at period II (12–24 h after imbibition), while $Cd^{2+}$ was more effective to inhibit germination at period I (0–12 h after imbibition) rather than at period II. The observed differences are likely to be due in part to selective uptake of different ions by the intact seed, because isolated embryos (without seed coat and endosperm) are more sensitive to both $Hg^{2+}$ and $Cd^{2+}$ at period I. We assessed interactive toxicity between heavy metals and non-toxic cations, and found that $Ca^{2+}$ was able to partially restore the inhibition of seedling growth by $Pb^{2+}$ and $Zn^{2+}$.

## Introduction

Some heavy metals, such as Cu, Zn and Ni, are essential micronutrients for plants, but are toxic to organisms at high concentrations (Munzuroglu and Geckil 2002). Plants are sometimes exposed to non-essential heavy metals, including $Hg^{2+}$, $Cd^{2+}$ and $Pb^{2+}$, that are present in soil and water naturally or as contaminants from human activities. Recent work has started to uncover molecular mechanisms underlying the uptake and transport of some heavy metal species (Howden et al. 1995; Blaudez et al. 2003; Song et al. 2003). However, it remains to be further investigated how essential and non-essential metal ions affect plant growth at different developmental stages under varying environmental conditions.

Seed is a stage in the plant life cycle that is well protected against various stresses. However, soon after imbibition and subsequent vegetative developmental processes, they become stress-sensitive in general. Therefore, seeds are thought to carefully monitor such external parameters as light, temperature and nutrient in order to maintain the protective state until external conditions become favorable for following developmental processes (Karssen 1982; Pritchard et al. 1993; Bungard et al. 1997). Although such critical

regulatory mechanisms are likely to operate in seeds at the onset of imbibtion, little is known about how stress tolerance is modulated at different phases of germination. Using transcriptomics and proteomics approaches, molecular and biochemical events in imbibed *Arabidopsis* seeds have recently been analyzed in detail (Gallardo et al. 2001, 2002; Ogawa et al. 2003; Yamauchi et al. 2004). Accumulating knowledge on the regulation of dormancy and germination in this species will allow us to examine how stress tolerance is modified in imbibed seeds in response to different signals.

As a first step to understand how heavy metals affect the ability of seed germination, we have examined the toxicity of essential ($Cu^{2+}$ and $Zn^{2+}$) and non-essential metal ions ($Pb^{2+}$, $Cd^{2+}$ and $Hg^{2+}$) in imbibed *Arabidopsis* seeds at different developmental stages. We have also assessed interactive toxicity between heavy metals and non-toxic cations during germination and seedling growth.

## Materials and methods

### *Plant materials and chemicals*

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used as wild-type in this study. To obtain seeds for germination tests, plants were grown on soil under continuous white light at 22 °C. Harvested seeds were stored at room temperature with 30% relative humidity for 6 months before the start of germination experiments. Heavy metals (provided as chloride salts) were purchased from Nacalai Tesque Inc. (Tokyo, Japan). All solutions were made in doubly distilled water ($ddH_2O$), and $ddH_2O$ was used as a control treatment.

### *Germination tests*

Dry seeds were washed with 0.02% Triton-X solution, rinsed with water twice (doubly-distilled water was used throughout this study), and then washed with the test solution twice (Yamaguchi et al. 1998). The seeds were placed on double-layered filter papers (3 mm, Whatman, Maidstone, UK) wetted with 0.7 ml $ddH_2O$ or test solutions in a 3.5-cm Petri dish. Petri dish were sealed and incubated under continuous white light at 22 °C. When test solutions were changed during incubation, the seeds were collected in a 1.5-ml tube, rinsed with $ddH_2O$, and then washed twice with the new test solution. The seeds were then incubated on filter papers as described above. Germination tests were carried out using triplicate samples (each containing 50–60 seeds). Seeds were scored as germinated when the breakage of seed coat was visible. Seedling development was regarded as being inhibited 6 d after imbibition if the seed coat was visibly broken (germination), but the embryo did not grow further.

Seed coats were removed carefully using a forceps within 30 min after imbibition on wet filter papers. The isolated embryos were incubated on filter papers without or with heavy metals (0–12 h or 12–24 h) under continuous white light. When metal ions were treated for the first 12 h, seed coats were removed in the presence of the metal ion.

## Results

### *The effect of heavy metals on seed germination and seedling growth*

Figure 1a shows the effect of heavy metals on germination of after-ripened *Arabidopsis* seeds under continuous white light at 22 °C. As reported for other plant species, $Cu^{2+}$, $Pb^{2+}$ and $Zn^{2+}$ were not very toxic for seed germination in *Arabidopsis* ($ID_{50}$ [50% inhibition] is >50 mM). In comparison, $Hg^{2+}$ ($ID_{50}$ = 0.7 mM) and $Cd^{2+}$ ($ID_{50}$ = 6 mM) inhibited germination at much lower concentrations. In the presence of heavy metals at certain concentrations, the radicle protruded from testa, but the embryo growth was arrested beyond this point. We examined the arrest of seedling growth by heavy metals 6 d after imbibition, while germination (breakage of seed coat) was scored at 2 d. Figure 1b illustrates that the inhibition of seedling growth occurs at lower concentrations of $Cu^{2+}$, $Pb^{2+}$, $Zn^{2+}$ and $Hg^{2+}$ that did not inhibit germination. In contrast, $Cd^{2+}$ exhibited toxicity to seed germination and seedling growth at similar concentrations. Interestingly, two of the essential metals used in this study

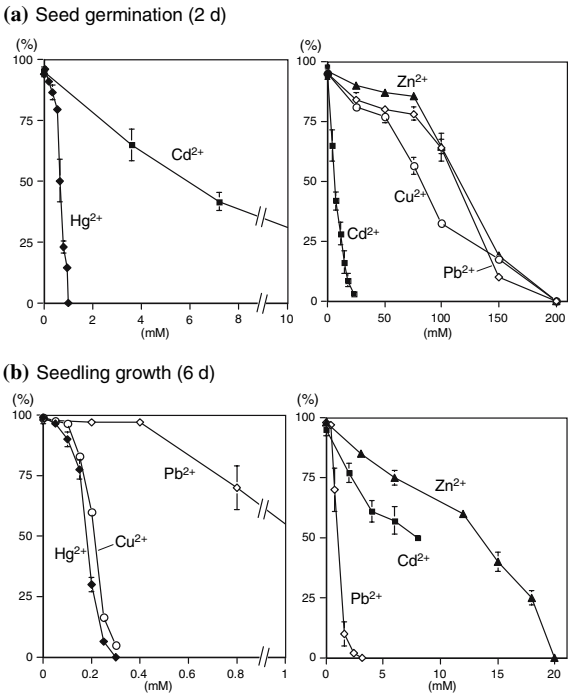


*Figure 1*. Seed germination (a) and seedling growth (b) of *Arabidopsis* in the presence of various heavy metals. (a) Seeds were scored as germinated when the breakage of seed coat was visible at 2 d after imbibition. (b) Seedling growth was regarded as being arrested if the seed germinated, but the embryo did not grow further within 6 d after imbibition. For clarity, the results are shown in two separate graphs with different concentration ranges in each panel. As a comparison, $Cd^{2+}$ in (a) and $Pb^{2+}$ in (b) are shown in both graphs.

($Cu^{2+}$ and $Zn^{2+}$) were not effective to inhibit germination, but did cause strong inhibition of seedling growth at relatively low concentrations (Figure 1b).

### *Heavy metal toxicity is altered developmentally during germination*

To examine if sensitivity to heavy metals changes developmentally during seed germination, the seeds were incubated with metal ions at period I (0–12 h) or period II (12–24 h) under continuous white light (Figure 2a). As shown in Figure 2b, seeds were significantly more sensitive to $Hg^{2+}$ at period II than period I, while $Cd^{2+}$ was evidently more effective in inhibiting germination at period I than at period II.

### *Heavy metal toxicity on dissected embryos*

Tissues covering the embryo, such as testa and aleurone (endosperm), may play an important role in protecting the embryo from heavy metal toxicity. To examine this hypothesis, seed coats were mechanically removed shortly (30 min) after imbibition, and the dissected embryos were incubated with heavy metals under continuous white light at period I (0–12 h) or period II (12–24 h) (Figure 3a). In contrast to intact seeds (Figure 2), isolated embryos were more sensitive to $Hg^{2+}$ at period I (Figure 3b), suggesting a protective role of surrounding tissues against $Hg^{2+}$ at period I. Other heavy metals, with the exception of $Cu^{2+}$, were more toxic to the growth of isolated embryos at period I (Figure 3b). Generally, isolated embryos were much more sensitive to heavy metals than intact seeds (Figures 1–3).

### *Interactive effects of non-toxic cations and heavy metals*

The toxicity of heavy metals to seed germination and seedling growth is known to be affected by other environmental factors, such as pH and availability of other nutrients (Kjar et al. 1998). But, the effect of non-toxic cations, such as $Ca^{2+}$, $Mg^{2+}$, $K^+$, $Na^+$, on the toxicity of heavy metals is unknown. Recent work has shown that $Cd^{2+}$ affects guard cell regulation by entering the cytosol via $Ca^{2+}$ channels, suggesting an interaction between the action of heavy metals and essential non-toxic cations (Perfus-Barbeoch et al. 2002). We therefore assessed interactive toxicity between heavy metals and non-toxic cations ($Ca^{2+}$, $Mg^{2+}$, $K^+$, and $Na^+$).

All cations examined in this study slightly increased the toxicity of $Hg^{2+}$ to seed germination, but did not affect the toxicity of $Cd^{2+}$ (data not shown). The toxicity of $Hg^{2+}$ was enhanced by the presence of cations used (the effect of $Ca^{2+}$ is shown in Figure 4). The effect of $Cd^{2+}$ and $Cu^{2+}$ on seedling growth was not influenced by cations at concentrations that we tested (data not shown). Interestingly, $Ca^{2+}$ was effective to reduce the toxicity of $Pb^{2+}$ and $Zn^{2+}$ (Figure 4); in the presence of 5 mM $Ca^{2+}$, the inhibition of early seedling development by $Pb^{2+}$ and $Zn^{2+}$ was partially restored. $Mg^{2+}$, $K^+$ and $Na^+$ at

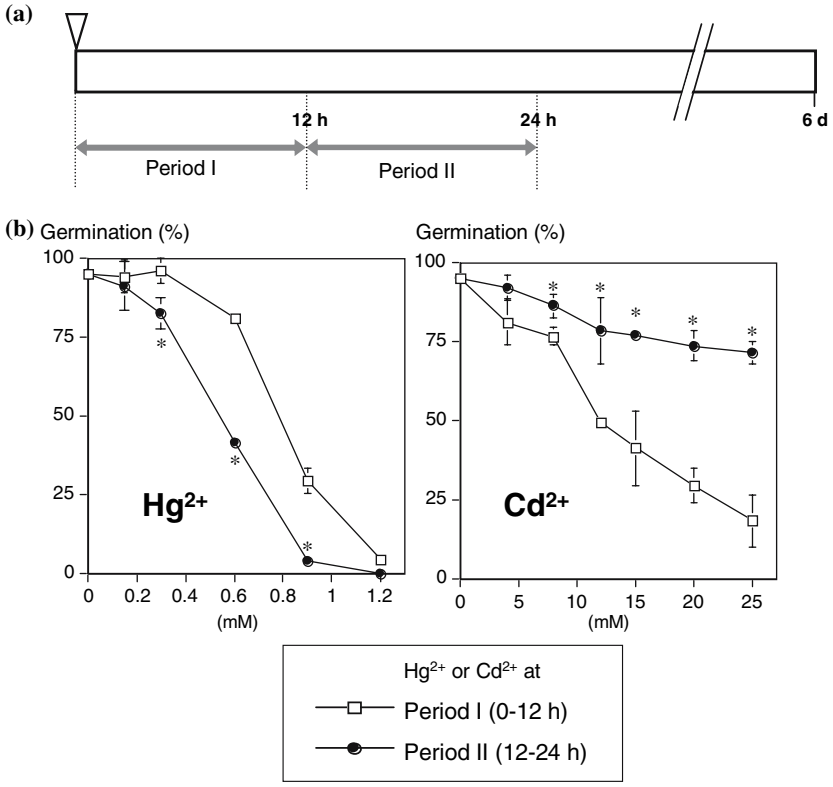


*Figure 2.* Developmental alterations in the toxicity of $Cd^{2+}$ and $Hg^{2+}$ to imbibed *Arabidopsis* seeds. (a) Diagram showing heavy metal treatments. The reverse triangle depicts the start of imbibition. Seeds were treated with heavy metals only at period I (0–12 h) or period II (12–24 h), as indicated by grey lines with arrowheads. Seed germination was scored at 6 d. (b) Germination percentage under conditions depicted in (a). Asterisks (*) represent significant differences between the two treatments at the same heavy metal concentration.

the same concentration did not show a similar effect.

## Discussion

In this study, we have examined the toxicity of selected heavy metals on seed germination and seedling growth in the model species *Arabidopsis*. In general, our results are consistent with previous reports regarding the effect of respective heavy metals. For example, $Cu^{2+}$ was not very toxic to seed germination, but it was to seedling growth (Fargasova 1994; Kjar et al. 1998; Xiong and Pahlsson 1998; Munzuroglu and Geckil 2002). $Hg^{2+}$ was commonly the most toxic metal for seed germination and seedling development. The spectrum and dose-response relationships of inhibitory effects on seed germination of various heavy metals in *Arabidopsis* are largely similar to those in cucumber (Munzuroglu and Geckil 2002). These results suggest that *Arabidopsis* is a useful model species to study molecular mechanisms for heavy metal toxicity during seed germination and seedling growth.

Our results support the idea that tissues covering the embryo play a role in selective penetration of different heavy metals into seeds. This was first suggested by the fact that seeds still germinated in the presence of high concentrations (~100 mM) of $Cu^{2+}$, $Pb^{2+}$ and $Zn^{2+}$, but the subsequent seedling growth (after the breakage of seed coat) was severely inhibited at much lower concentrations of these heavy metals. Our results also showed that isolated embryos were much more sensitive to heavy metals than intact seeds. This was observed most clearly for $Cu^{2+}$. Seed germination was completely inhibited by 200 mM $Cu^{2+}$ at 24 h (Figure 1). However, after the transfer of seeds to $ddH_2O$ from 200 mM $Cu^{2+}$, they still germinate nearly 100% (data not shown). In contrast,

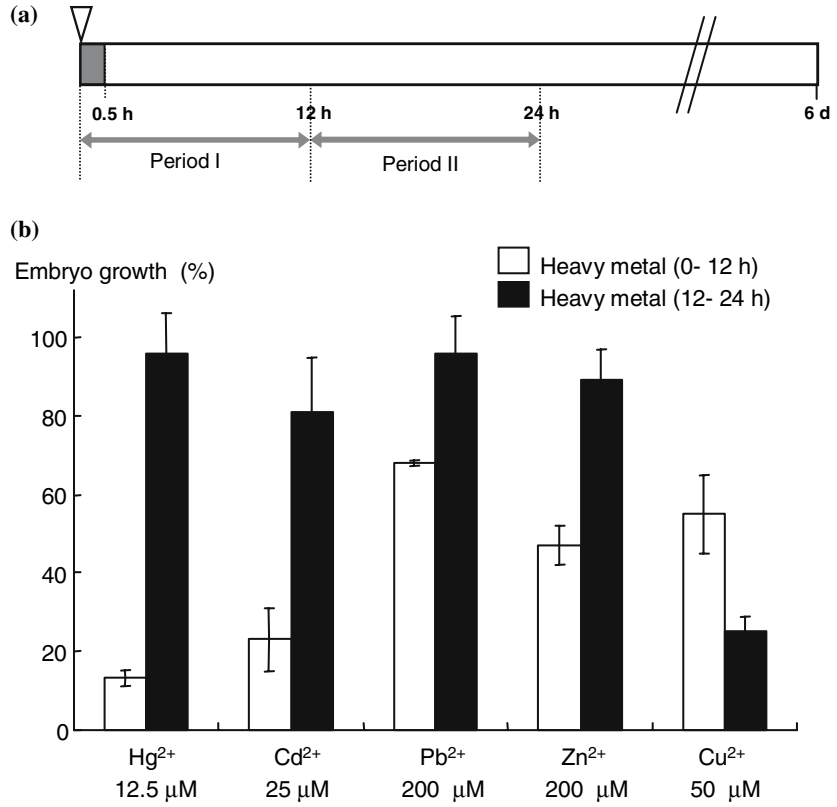


*Figure 3*. Growth of isolated embryos in the presence of heavy metals. (a): Diagram showing heavy metal treatments. The reverse triangle depicts the start of imbibition. The grey box indicates the duration required for embryo dissection at the beginning of imbibition (0.5 h). Isolated embryos were treated with heavy metals only at period I (0–12 h) or period II (12–24 h), as indicated by grey lines with arrowheads. Embryo growth was scored at 6 d. (b): The effect of heavy metals at period I (white bars) or period II (black bars) on the growth of isolated embryos. When the development of embryos was not visibly observed at all within 6 d after imbibition, it was regarded as no growth.

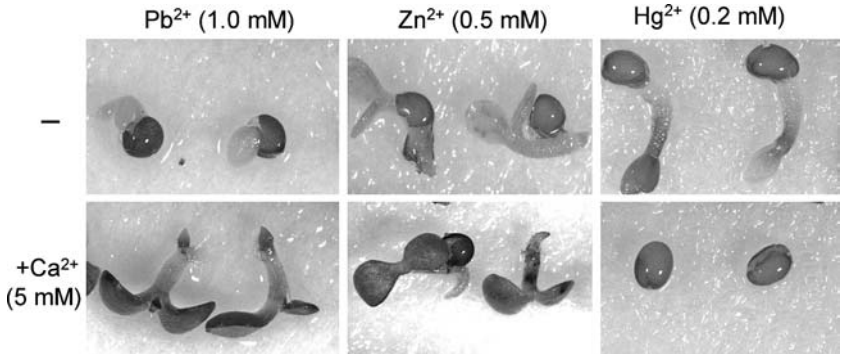


*Figure 4*. Effect of $Ca^{2+}$ on the heavy metal toxicity. *Arabidopsis* seeds were imbibed with heavy metals in the absence (–) or presence of 5 mM $CaCl_2$ ($+Ca^{2+}$). Photos were taken 6 d after the start of imbibition.

viability of isolated embryos was lost at 0.1 mM $Cu^{2+}$, indicating that tissues covering the embryo effectively block $Cu^{2+}$ during seed imbibition.

As shown in Figure 2, $Cd^{2+}$ displayed clearly stronger toxicity to seed germination at period I than at period II. Similar results were obtained

using seeds of another *Arabidopsis* ecotype Landsberg *erecta* (data not shown).

This study revealed a unique role of $Ca^{2+}$ in restoring the growth defect caused by $Pb^{2+}$ and $Zn^{2+}$. It has been thought that heavy metals are taken up by the cell through metal transporters. Several such transporter proteins have recently been identified (for review, see Clemens 2001). However, multiple pathways are likely to exist for the uptake of most metal ions (Clemens 2001), and it is at moment not clear which transporters contribute to the uptake of $Pb^{2+}$ and $Zn^{2+}$ in seeds and how $Ca^{2+}$ affected their toxicities.

In summary, we have determined the toxicity of selected heavy metals on seed germination and early seedling growth in the model plant species *Arabidopsis*. Our results suggest that the toxicity of heavy metals to *Arabidopsis* seeds is dependent on the physiological state of seeds, and that selective permeation of different metal ions through tissues surrounding the embryo is in part responsible for determining the toxicity.

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