Lee *et al. Applied Biological Chemistry* (2021) 64:87
https://doi.org/10.1186/s13765-021-00660-z

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The Korean Society for Applied Biological Chemistry

ARTICLE Open Access

Assessing the effects of accumulated Cd(II) on seed germination and root development of *Arabidopsis thaliana*

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Abstract

In this study, *Arabidopsis thaliana* was used as a model system to assess the toxic effects of cadmium on plant development and growth. The germination and growth of *A. thaliana* was inhibited by Cd(II), and the inhibitory effect was dosage-dependent. The significant decrease of germination rates and root growths of *A. thaliana* were observed from 50 mg/L and 25 mg/L of $CdCl_2$, respectively. Although both shoot and root growths were suppressed by Cd(II), root developments were more sensitive to Cd(II) than shoot developments, as evidenced by shoot growths observed over 50 mg/L of $CdCl_2$. In the concordance to this result, it was also observed that the expression of *DR5::VENUS*, a visual marker of auxin response, was dependent on the Cd(II) concentration and was strongly reduced from 5 mg/L of $CdCl_2$. In addition, the *E. coli*-based biosensors were employed to quantify accumulated Cd(II) in plants to understand the correlation between toxic effects and Cd(II) in plants. As a result, it was revealed that 0.012 mg/g and 0.138 mg/g of Cd(II) in dried plants were corresponded to the concentration inhibiting root developments and root growths, respectively. Although it needs further investigations, the findings play a significant role in assessing the toxic effects of Cd(II) based on the relationship between the toxic effects and accumulated Cd(II) concentrations in plants.

Keywords: *Arabidopsis thaliana*, Auxin, Biosensors, Cadmium, Root development

Introduction

Heavy metals/metalloids have a negative impact on the environment; this impact has been worsening owing to rapid industrial development and urbanization. Although heavy metals occur naturally on Earth by rock formation processes, large amounts of heavy metals have been produced and released into the environment, especially in aquatic and soil environments that are closely associated with the biosphere [1–3]. The influx of heavy metals from agriculture, mining, and manufacturing, and waste industries into the soil and aquatic environments is a major environmental concern. Cadmium (Cd) exhibits toxic effects on human health because of its high solubility, which facilitates easy transport to humans via food chains [4–6].

Previous studies have indicated that heavy metals act as stress factors for plants. Heavy metal accumulation in plants affects the physiological and genetic plant signaling systems, thereby adversely affecting germination, growth, and production [7, 8]. Additionally, Cd accumulation in seeds induces lipid oxidation, thus, causing biomass loss and inhibiting germination, shoot elongation, and activities of enzymes such as alpha-amylase and invertase [9, 10]. Furthermore, it induces overexpression of a thioredoxin-dependent enzyme, Gpx, which leads to a decrease in the activity of glutathione reductase and affects the functioning of mitochondria owing to reduced thiol levels [11]. Moreover, Cd inhibits the activity of Fe (III) reductase, which is associated with photosynthesis, and affects the permeability of plasma membrane,

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thus, reducing the water content [1, 12]. In *Arabidopsis thaliana*, Cd is majorly accumulated in roots (89%), thus, reducing the formation of root hair, lateral roots, and biomass, whereas Cd accumulated in leaves (11%) induces chlorosis and size reduction. Mostly the accumulated Cd exists in a water-soluble form, but it forms a complex with bioligands, glutathione, cysteines, and phytochelatins [13].

When Cd released in the environment enters plants, multiple responses are triggered to neutralize toxicity. The toxic effects of Cd on plants have been studied extensively. Subsequently, many genes and enzymes responsible for inducing Cd response mechanisms were identified [14, 15]. In addition, the mechanisms of Cd uptake in plants, and its transportation and distribution have been investigated previously [16, 17]. However, the relationship between the accumulated Cd concentrations in plants and the toxic effects was not considered in most studies that investigated the physiological and genetic effects of external Cd concentrations on plants, rather than those of accumulated Cd concentrations. Since the Cd mobility and accumulation rate varies depending on environmental conditions and plant species, studying the toxic effects of accumulated Cd in plants is significant. In this regard, the *Escherichia coli* (*E. coli*) cell-based biosensors were employed to measure the accumulated Cd in plants. Bacterial cell-based biosensors had been investigated and applied to quantify toxic materials including heavy metals and chemicals in environmental systems [18–20]. Although the accuracy and precision for detection were relatively low compared to instrumental-based analysis, bacterial cell-based biosensors were much less expensive and much faster for analysis. Additionally, the ability to quantify bioavailable portion of toxic materials was the most advantageous aspect of bacterial cell-based biosensors [21, 22].

In this study, we aimed to assess the adverse effects of Cd on plant germination and root development along with the variations in plant hormone levels using *Arabidopsis thaliana* exposed to different Cd concentrations. Furthermore, the accumulated Cd concentrations in plants were determined by *Escherichia coli*-based biosensors to identify its correlation with physiological effects on plants rather than exposed concentrations.

Materials and methods

Plant materials, growth, and treatment

Arabidopsis thaliana ecotype Columbia (Col-0) was used as a control plant in this study. Seeds were surface-sterilized using 70% ethanol and plated on half-strength Murashige and Skoog (1/2 MS) solid media. After 3 days of vernalization at 4 °C in darkness, plants were grown in a growth chamber with a light regime of 16 h/8 h (light/dark) at 23 °C. Subsequently, the seedlings were transferred into the soil for further tests. Later, 1 mM of Cd(II) stock solution was prepared by dissolving $CdCl_2$ (Sigma Aldrich) with water for the $CdCl_2$ treatment. The soils, named as Hanareum Horticultural Bed Soil, for shoot growth assay were commercially available and purchased from Shinsung Mineral Co. Ltd. (Korea).

Germination assay

Seeds of wild-type plants (Col-0) were placed on the 1/2 MS solid media containing 0, 10, 25, 50, and 100 mg/L of Cd(II) and germinated in a growth chamber with 16 h/8 h (light/dark) conditions at 23 °C. After a 7-days incubation period, seed germination was monitored, and the germination ratio (total number of seeds germinated/total number of seeds planted) was quantified. The experiments were conducted in triplicates.

Root growth assay

To analyze the effect of Cd(II) on root growth, the seeds of the wild-type plants were placed on the 1/2 MS solid media supplemented with 0, 10, 25, 50, and 100 mg/L of Cd(II). The seeds were germinated in a growth chamber with 16 h/8 h (light/dark) conditions. After an incubation period of 7 days, root growth in plants was recorded and quantified using ImageJ program. Furthermore, to analyze the effect of Cd(II) on lateral root formation, the wild-type plants were grown on the 1/2 MS solid media supplemented with 0, 1, 5, and 10 mg/L of Cd(II) for 14 days. The experiments were conducted in triplicates.

Shoot growth assay

To analyze the effect of Cd(II) on shoot growth, the wild-type plants were grown in soil for 3 weeks. MS liquid media including 100 mg/L of $CdCl_2$ were painted on the leaf surface each day for the Cd(II) treatment. After 7 days of the treatment, shoot growth was recorded and quantified. The experiments were conducted in triplicates.

Visualization of auxin response

To visualize the fluorescent signals in auxin response, *DR5::VENUS* plants, visual markers of auxin response [17, 23] were grown in Cd(II)-treated and -untreated conditions for 7 days. The whole roots of the plants were stained with propidium iodide (PI) solution (20 μg/mL) for 2 min, after which the roots were mounted on glass slides in double-distilled water (ddH_2O). Fluorescence was visualized at wavelength range of 591–635 nm and 505–530 nm for PI and VENUS signals, respectively.

Quantification of accumulated cadmium in plants

The plants grown in the MS agar plates with varying Cd concentrations were collected after a 2-week incubation period. The plants were washed with distilled water and buffer intensively and dried at 60 ℃ for 3 days. Subsequently, 10 mg of dried plants were ground with 1000 μL of metal-free water to prepare whole plant samples. The water-soluble extracts were prepared by centrifugation of the whole plant samples at 14 000 rpm for 20 min. A Cd specific *E. coli*-based biosensor was employed to quantify the Cd(II) concentrations in *A. thaliana* grown in Cd(II) MS agar plates. The biosensor has been used previously, and the bioassay using this biosensor in the present study was conducted following the previously reported procedures with minor modification [24, 25]. Briefly, *A. thaliana* samples were subjected to a fresh culture of biosensor cells at an optical density of 0.4 at 600 nm (OD_{600}). The fluorescent signals of biosensors were determined by the FC-2 spectrophotometer after 1 h of exposure. The fluorescent signals were converted to induction coefficient values defined as eGFP signal from Cd(II) exposed samples/eGFP signal from control samples. The accumulated Cd(II) concentrations in plants were determined by a standard curve acquired using a known Cd(II) concentration range of 0–100 μg/L.

Statistical analysis

When the minimum difference between the control and the treatment means was statistically significant, Dunnett Program (Ver 1.5) was used for statistical analysis of all data obtained in this study. The analysis was performed with a 95% significance level ($p < 0.05$).

Results

Inhibitory effect of Cd(II) on seed germination of *Arabidopsis thaliana*

The effects of Cd(II) on seed germination were analyzed using more than 300 *A. thaliana* seeds grown for 7 days in the MS solid media supplemented with 0, 10, 25, 50, and 100 mg/L of $CdCl_2$; subsequently, the germination rates were quantified (Fig. 1). Although the MS media with 10 mg/L of $CdCl_2$ did not demonstrate evident effects on the germination rate compared with the control MS media without $CdCl_2$, the germination rates gradually decreased with increasing Cd(II) concentrations. The germination rate in the plants grown in the MS media supplemented with 25 mg/L of $CdCl_2$ decreased significantly. The inhibitory effect of Cd(II) was dosage-dependent. The germination rates decreased in the plants grown in the presence of 50 mg/L (55%) and 100 mg/L of $CdCl_2$ (21%) (Fig. 1F), thus, suggesting that seed germination was negatively affected by Cd(II).

Differences in the root and shoot growths upon Cd(II) exposure

To understand the effect of Cd(II) on the overall plant growth, we analyzed the shoot and root developments of *A. thaliana* grown in the MS solid media with 0, 10, 25, 50, and 100 mg/L of $CdCl_2$ for 7 days (Fig. 2). Compared with the plants grown in the control MS solid media, the root developments in the Cd(II)-treated plants was suppressed. The root lengths of the plants grown in the media with 10 mg/L of $CdCl_2$ was approximately 60% of the control plant roots, whereas the root developments were significantly suppressed in the plants grown in the media including 25, 50, and 100 mg/L of $CdCl_2$. The plants grown in 25 mg/L $CdCl_2$ conditions developed extremely small roots (< 3 mm in length), while those grown in 50 mg/L or 100 mg/L $CdCl_2$ conditions almost failed to develop roots (Fig. 2A, B). Similar to the effect on roots, Cd(II) negatively affected shoot developments depending on the dosage, but the suppression in shoot developments was lower than that in root development. In contrast to root development, shoot developments did not significantly reduce in conditions of 10 mg/L or 25 mg/L of $CdCl_2$. The lengths of leaves of the plant grown in these $CdCl_2$ concentrations were similar or slightly less than that of the untreated control plants (Fig. 2C, D). Significant decrease in shoot developments was observed at concentrations exceeding 50 mg/L of $CdCl_2$; approximately 55% and 28% decrease in the lengths of plant leaves were observed in conditions with 50 mg/L and 100 mg/L of $CdCl_2$ respectively, compared with that in the lengths of the leaves of the control plants (Fig. 2D). These findings indicated that both root and shoot developments were negatively regulated by Cd(II), but root developments was more sensitive to Cd(II). To further verify these results, we treated the wild-type plants grown under normal growth conditions with 100 mg/L of $CdCl_2$ and analyzed the changes in shoot and root developments (Fig. 3). Exogenous treatment by painting 100 mg/L of $CdCl_2$ did not affect shoot developments but completely suppressed root developments. Thus, the inhibitory effects of Cd(II) on root developments were stronger than those on shoot developments.

Effects of Cd(II) on lateral root formation

Cd(II) affected lateral root formation along with inhibition of root development (Fig. 4). *A. thaliana* roots grown in the control MS media for 2 weeks formed approximately five lateral roots, with a 1.5-cm lateral root forming zone. Exogenous treatment with $CdCl_2$ reduced the number of lateral roots depending on the dosage.

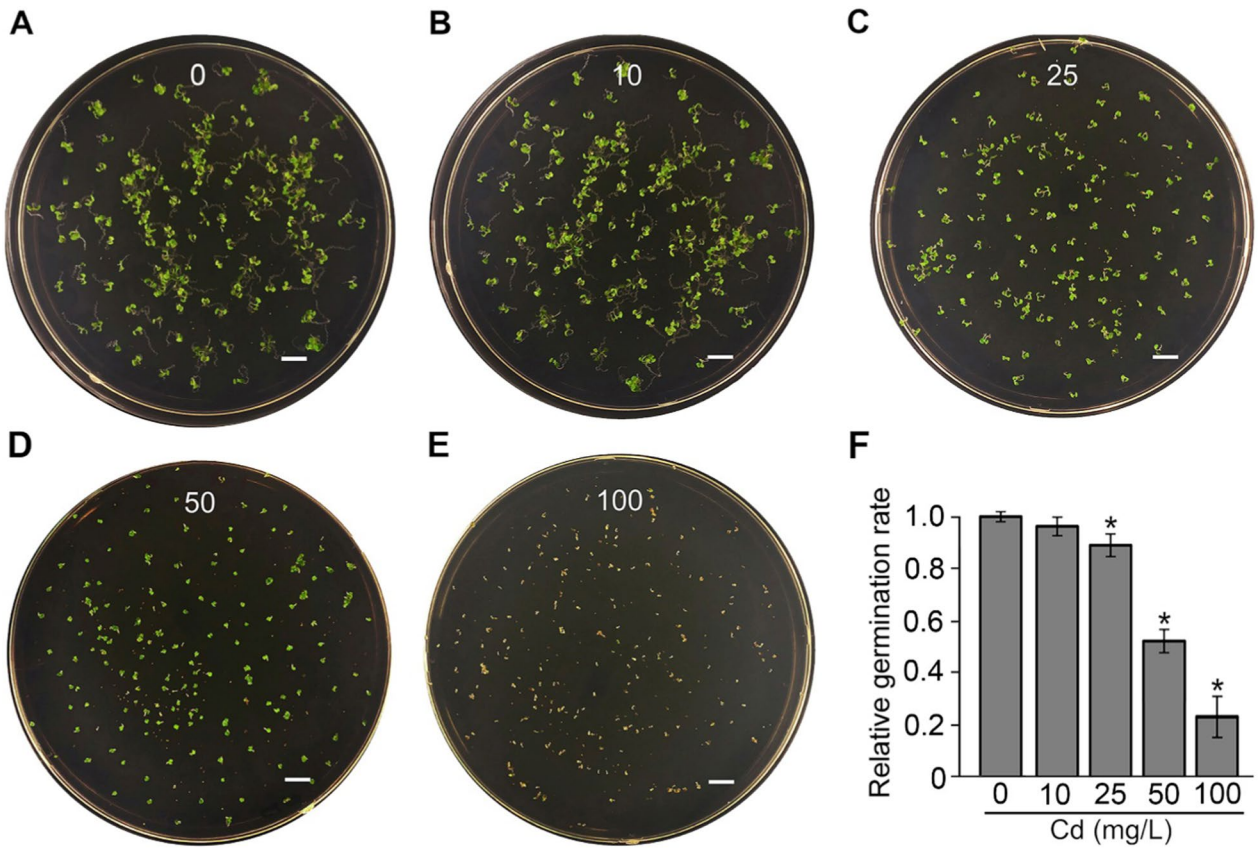

Fig. 1 Effect of Cd(II) on germination. **A–E** Images of plants grown in the Murashige and Skoog (MS) solid media with 0, 10, 25, 50, and 100 mg/L of $CdCl_2$ for 7 days. **F** Quantification of the germination rates (Number of germinated seeds/total number of seeds planted) in these plants (n > 300). Error bars indicate standard deviation (SD). Asterisks indicate statistically significant differences between the corresponding samples and their control (*p* value < 0.01, *t*-test). Scale bars = 1 cm

Consequently, the size of the lateral root forming zone gradually decreased with increasing $CdCl_2$ concentration, with approximately 1, 0.4, and 0.3 cm zone sizes for 1, 5, and 10 mg/L of $CdCl_2$, respectively (Fig. 4A–D). These results indicated that Cd(II) affected lateral root formation and apical root growth. To confirm these findings, we further monitored the effect of Cd(II) exposure on plant hormone concentration.

Changes in the expression of plant hormone

Auxins significantly control apical root growth and lateral root formation [26, 27]. Therefore, we hypothesized that Cd(II) affects auxin response in *A. thaliana* roots since Cd(II) suppressed both apical root growth and lateral root formation. We visualized the auxin response in *DR5::VENUS* plants by monitoring the green fluorescent signals that correspond to auxin response [23]. *A. thaliana* roots grown under normal growth conditions strongly exhibited green fluorescent signals in root tips and vascular tissues. Cd(II) negatively affected the signal intensity in root tips and vascular tissues (Fig. 4E) based on the dosage. The plant roots in 10 mg/L of $CdCl_2$ strongly exhibited reduced green fluorescent signals compared with the untreated control plant roots. Thus, Cd(II) suppressed both apical root growth and lateral root formation by negatively regulating auxin response.

Cd(II) accumulation in *Arabidopsis thaliana*

Quantification of Cd(II) in plants is crucial to understand its uptake rate and to assess the association between the accumulated Cd(II) and physiological interferences. *A. thaliana* specimens grown on MS agar plates with varying Cd(II) concentrations were washed intensively to rule out the interference of residual Cd(II) on the root surface. Plant specimens were prepared as water-soluble fractions and whole plant samples and subjected to *E. coli* cell-based bioreporters for quantification. The Cd(II) concentrations in plants were determined using the equation acquired from the standard curve from fluorescence spectrophotometer (Fig. 5). As shown in

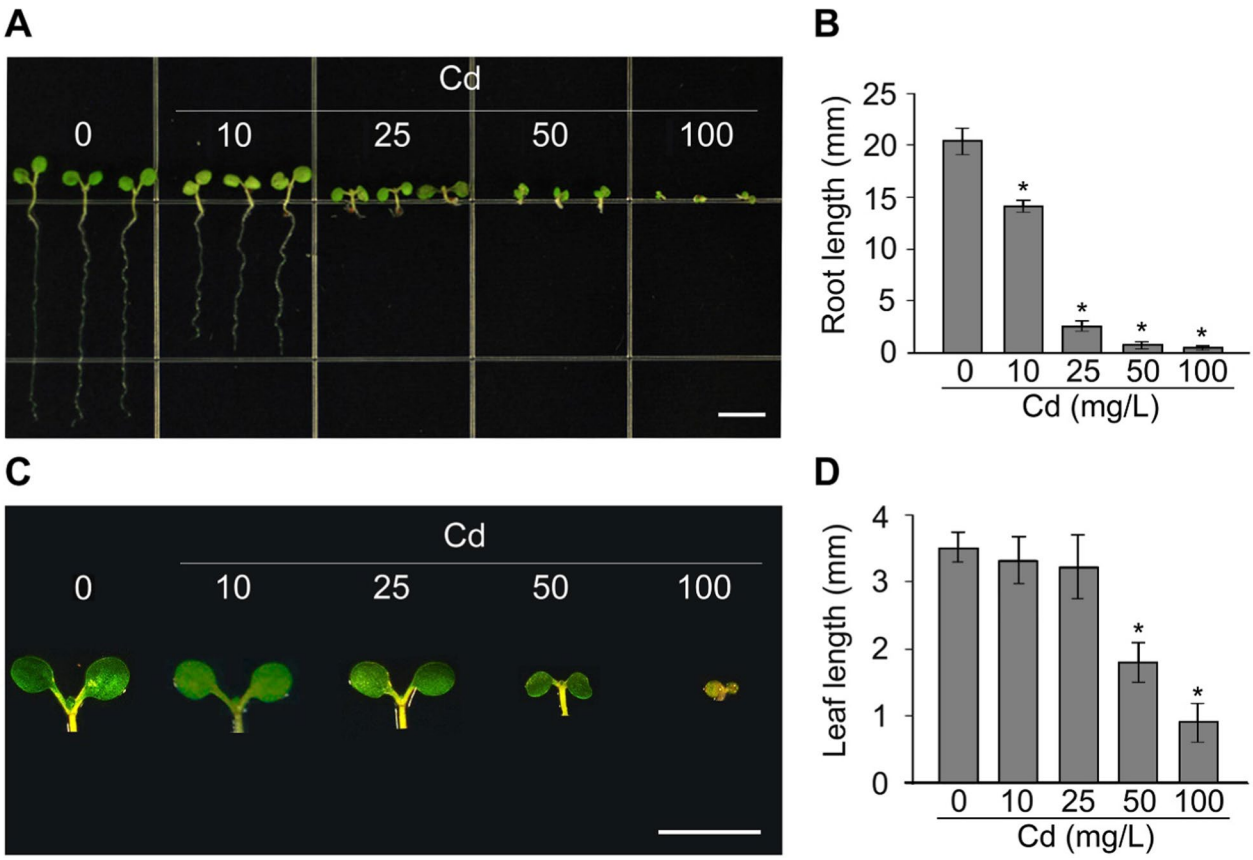

Fig. 2 Effect of Cd(II) on root and shoot growth. **A** Root growth in the plants grown in the MS solid media with 0, 10, 25, 50, and 100 mg/L of $CdCl_2$ for 7 days. **B** Quantification of root length of these plants (n > 50). **C** Shoot growth in the plants grown in the MS solid media with 0, 10, 25, 50, and 100 mg/L of $CdCl_2$ for 7 days. **D** Quantification of leaf length in these plants (n > 50). Error bars indicate SD. Asterisks indicate statistically significant differences between the corresponding samples and their control (p value < 0.01, t-test). Scale bars = 0.5 cm

Table 1, the accumulated Cd(II) concentration proportionally increased with the external concentration; similarly, the increase in the Cd(II) uptake rates depended on the external concentration. The Cd(II) uptake rates in plants exposed to 10 mg/L of Cd(II) was approximately 6.3 times higher than those in plants exposed to 5 mg/L (Table 1). Moreover, the Cd(II) concentration in the water-soluble extract was approximately 2–4 times less than that in the whole plant samples possibly because of the Cd(II) physicochemical properties, such as solubility, that affect the extraction efficiency. To address the accumulated Cd(II) effects, the total plant Cd(II), not the soluble Cd(II) concentration, was considered for further discussion. Root development was inhibited at 0.012 mg of accumulated Cd(II) per gram of dried plant. The root length decreased by approximately 17%, whereas the lateral root formation and zone size decreased by 30% and 29%, respectively. Although the inhibitory effect of the accumulated Cd(II) on the lateral root formation was low, the size of lateral root zone was severely inhibited at concentrations exceeding 0.138 mg/g of accumulated Cd(II). Root growth was inhibited by approximately 30% and 60% at 0.138 mg and 0.877 mg of accumulated Cd(II) per gram of dried plants, respectively. Despite the importance of risk assessment of environmental Cd(II) on plants, misinterpreting the risks is possible while considering the external Cd(II) concentration only, without accounting for the Cd(II) physicochemical properties and environmental factors. Therefore, understanding the association between the adverse effects and accumulated Cd(II) in plants is significant for conducting highly accurate risk assessments.

Discussion

The toxic effects of heavy metals including Cd(II) on plants had been investigated for a long time. It had been reported that heavy metals played as stresses to induce inhibitions of seed germination and root developments as well as decrease the productivity [28, 29]. Cd(II), one of toxic heavy metals persistent in environmental

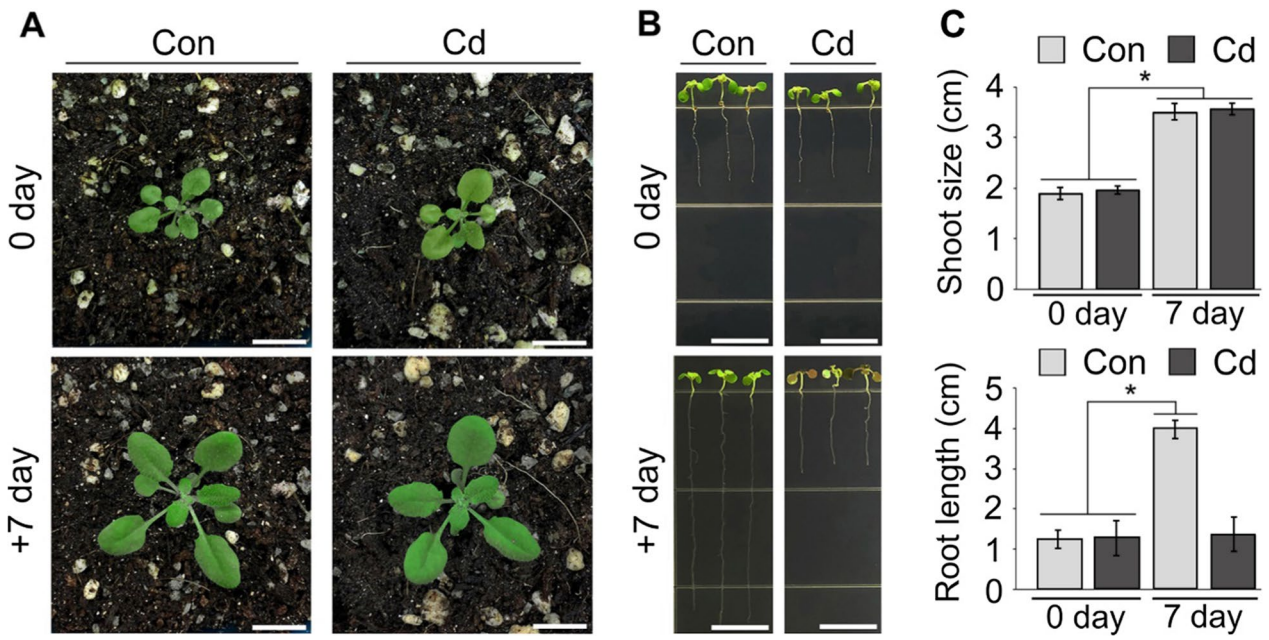

Fig. 3 Differences in the root and shoot growths due to Cd (II). **A** Images showing changes in the shoot growth of the plants treated (Cd) or untreated (Con) with $CdCl_2$. For the $CdCl_2$ treatment, plants grown in soil for 3 weeks were painted with the MS liquid media including 100 mg/L of $CdCl_2$ every day. **B** Images showing changes in the root growth of the plants treated (Cd) or untreated (Mock) with $CdCl_2$. For the $CdCl_2$ treatment, 7 days-old plants grown in the normal MS solid media were transferred to the MS solid media including 100 mg/L of $CdCl_2$ and grown for 7 days. **C** Quantification of shoot ($n > 20$) and root growth ($n > 60$) in these plants. Error bars indicate SD. Asterisks indicate statistically significant differences between the corresponding samples and their control (p value < 0.01, t-test). Scale bars = 1 cm

systems, had been considered as harmful materials and regulated strictly because of its toxicity and tendency to bioaccumulate on and to living organisms [6, 30]. It had been reported that Cd induced radial swelling of root tips, inhibits chlorophyll biosynthesis, and interrupts the uptake of essential elements as well as the inhibitions of seed germination and root developments [31, 32]. Although it was well studied about adverse effects of Cd(II) on plants, most of studies were focused on the amount of Cd(II) in exterior rather than in plants. Since the accumulation rates of Cd(II) were varied upon physiological properties of plant species, the correlation study between Cd(II) toxicity and accumulated amounts would be valuable for risk assessments. In this regard, we investigated adverse effects of Cd(II) exposure on physiological properties as well as correlated them with accumulated Cd(II) determined by *E. coli* cell-based biosensors.

Results here indicate that the inhibitions of seed germinations, root growths and developments were dependent on Cd(II) concentration, while critical concentrations showing significant inhibitions were different from each biological pathway. The dramatic reductions on seed germination rates and root growth were observed at 50 mg/L and 25 mg/L of $CdCl_2$, respectively. These findings were consistent of previous studies on *Leucaena leucocephala*, wheat, bean, and alfalfa, which demonstrated varying effects of heavy metals on seeds and roots [9, 33, 34]. Thus, it was inferred that adverse effects of Cd(II) were played differentially on different biological pathways.

Generally, root developments were more sensitive to toxic effects of heavy metals, and it would be related to the distribution of accumulated heavy metals in plants. Although the Cd(II) taken up by roots was transported to shoots, the amounts distributed in roots were much more than other places [35, 36]. In this regard, it was reasonable the negative effects of Cd(II) on plant shoot development were comparatively weak than on root developments (Fig. 2). These differences were further confirmed by comparing the root and shoot developments in plants grown with and without Cd(II). The shoot size remained unchanged after 7 days in both the treatments, but root development was completely inhibited in the plants grown in soil with Cd(II) (Fig. 3). Since the roots were more sensitive to toxic effects of Cd(II), the effects on lateral root formation were investigated. As shown in Fig. 4, the lateral root numbers and size of lateral root zone were decreased upon Cd(II) concentration as well as the length of primary roots. This finding led to investigate the level of auxin upon Cd(II) exposure because it is known to regulate root developments in

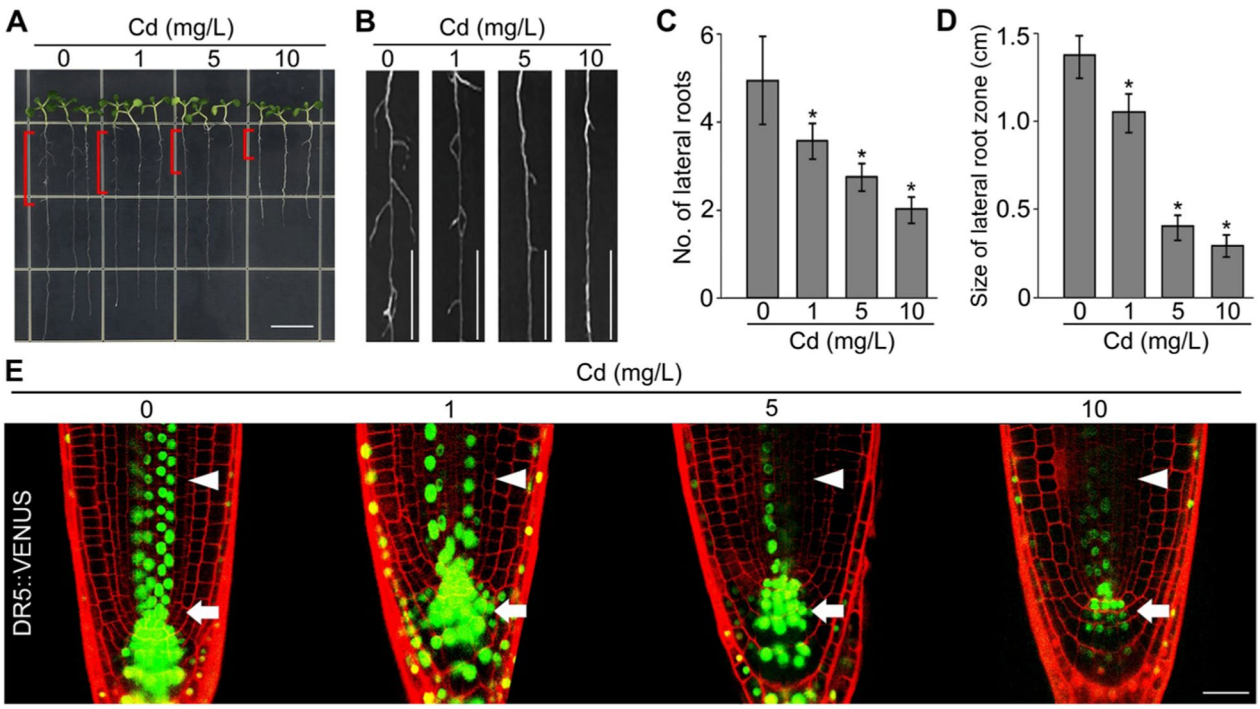

Fig. 4 Effect of Cd(II) on lateral root formation. **A**, **B** Images showing lateral root formation in the plants grown in the MS solid media with 0, 1, 5, and 10 mg/L of $CdCl_2$ for 2 weeks (**A**), and their high magnification images (**B**). Red lines in (**A**) indicate lateral root forming zones. **C**, **D** Quantification of the number of lateral roots (**C**) and the size of lateral root forming zone in these plants (**D**) (n > 30). Error bars indicate SD. Asterisks indicate statistically significant differences between the corresponding samples and their control (p value < 0.01, t-test). **E** Visualization of green fluorescent signals in *DR5::VENUS* grown in the MS solid media with 0, 1, 5, and 10 mg/L of $CdCl_2$ for 1 week. Green and red fluorescent signals indicate VENUS and propidium iodide signals, respectively. Arrows and arrowheads indicate the auxin response in root tips and vascular tissues, respectively. Scale bars = 1 cm in (**A**), 0.5 cm in (**B**), and 25 μm in (**E**)

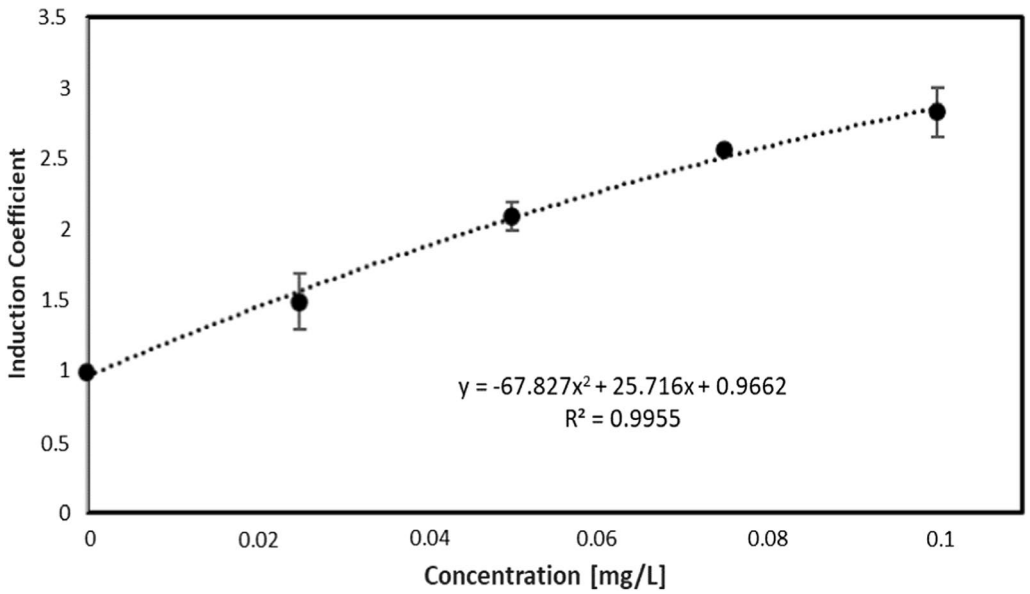

Fig. 5 Standard curve for quantifying the accumulated Cd(II) concentration in plants. The known Cd(II) concentration of ranging from 0 to 0.1 mg/L was spiked to *E. coli*-cell based biosensor and the induction coefficient values were determined after 1 h of exposure. The induction coefficient values were plotted against the Cd(II) concentration and the equation was obtained by a hyperbolic curve fit with $R^2 = 0.995$

Table 1 The accumulated Cd(II) concentration in plants depending on the external $CdCl_2$ concentration and its association with the inhibitory effects on physiological properties in root development

External $CdCl_2$ concentration (mg/L)	Amount of Cd(II) in plant (mg/g of plant)		Root length (%)	Lateral root formation (%)	Lateral root forming zone (%)
	Water soluble	Whole plant			
0	ND	ND	–	–	–
1	0.008 ± 0.002	0.012 ± 0.005	17	30	29
5	0.032 ± 0.010	0.138 ± 0.039	34	40	71
10	0.365 ± 0.074	0.877 ± 0.195	62	50	78

Root length and lateral roots were analyzed after 2 weeks of exposure, and auxin response was determined after 1 week of exposure

ND not detectable

plants. The auxin levels in *DR5::VENUS* plants grown at the same experimental conditions were analyzed, and it clearly decreased at 5 mg/L of Cd(II) (Fig. 4E). Compared to control, the localizations and intensity of fluorescence were disrupted and diminished upon Cd(II) exposure. As mentioned above, the adverse effects of Cd(II) on root developments have been intensively studied and the toxic mechanisms had been revealed. Nonetheless, most of studies were focused on the amount of Cd(II) present exterior of plants rather than the amount inside of plants. However, it would be worthy to know the correlation between toxic effects and the accumulated Cd(II) in plants.

As pointed out, the uptake of heavy metal(loid)s is dependent on not only environmental availability but also plant uptake mechanisms. Thus, it is important to quantify accumulated heavy metal(loid)s, which is the portion causing adverse effects on plants, and to achieve accurate risk assessment. In this regard, we used Cd(II)-specific *E. coli* cell-based bioreporter employing zinc responsive operon (*znt*-operon) reported previous was used to analyze the accumulated Cd(II) in *Arabidopsis* [24, 25]. As described in "Results" section, the accumulated Cd(II) in plants was proportional to exposed amount of Cd(II), and uptake rates were also increased (Table 1). Additionally, it was noticed that 0.012 mg of Cd(II) per 1 g of dried plants showed the inhibition of root lengths, lateral root formation and size of lateral root forming zone. Although the toxic effects of Cd(II) on plant growth was investigated intensively during past few decades, it had not been revealed the correlation between accumulated Cd(II) and toxic effects. We believed that the study showing the relationship between toxic effects and the amount of accumulated heavy metals would be invaluable to understand differential toxic effects on different plant species as well as to achieve accurate risk assessments.

Acknowledgements
This study was supported by the Korea Agency for Infrastructure Technology Advancement (KAIA) Grant funded by the Ministry of Land, Infrastructure, and Transport (Grant 21UGCP-B157945-02), and by the BioGreen21 Agri-Tech Innovation Program Rural Development Administration, Republic of Korea (Project No. PJ01567301).

Authors' contributions
Conceptualization: GJ, YY; methodology: YL, JJ; formal analysis and investigation: YJ, HK; writing-original draft preparation: YY; writing-review and editing: GJ, YY. All authors read and approved the final manuscript.

Availability of data and materials
All data generated or analyzed during this study are included in this manuscript.

Declarations

Competing interests
The authors declare no conflict of interest.

Received: 19 October 2021 Accepted: 7 December 2021
Published online: 18 December 2021

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