

A PILOT STUDY TO DETERMINE THE SUBSTRATE THRESHOLD FOR HEAVY METAL TOXICITY IN GROUNDCOVER PLANTS USED IN URBAN LANDSCAPES

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Abstract. The objects of this pilot study were to determine the substrate threshold concentration for heavy metal toxicity, which is the highest permissible content in the substrates used in urban landscaping, and the maximum possible accumulation of heavy metals in shoots of *Pachysandra terminalis* and *Vinca minor* grown on substrate mixed with cadmium (Cd), lead (Pb), or zinc (Zn) for 90 days. Plants were transplanted into plastic containers filled with substrate, which had been previously treated with Cd (0, 25, 50, or 100 mg·kg⁻¹), Pb (0, 250, 500 or 1,000 mg·kg⁻¹), and Zn (0, 500, 1,000, or 2,000 mg·kg⁻¹). The shoot dry weights of *P. terminalis* and *V. minor* were affected by the heavy metals in the substrate with the greatest degree by Cd. A 25 mg·kg⁻¹ of Cd caused a reduction of about 63% and 30% of shoot dry weights of *P. terminalis*, and *V. minor*, respectively, compared to controls (Cd 0 mg·kg⁻¹). There was no reduction in *P. terminalis*, and *V. minor* grown under both Pb 250 mg·kg⁻¹ and 500 mg·kg⁻¹ treatments. Under the Pb 1,000 mg·kg⁻¹ treatment, a 3.7% of dry weight loss observed in *P. terminalis* compared to control (Pb 0 mg·kg⁻¹); however, no reduction was found in *V. minor*. Zn-treated *V. minor* had no reduction of dry weight compared to controls (Zn 0 mg·kg⁻¹), whereas in *P. terminalis*, about 11%, 26%, and 52% reductions were observed under Zn 500, 1,000, and 2,000 mg·kg⁻¹ treatments, respectively. These results reveal that *V. minor* is more tolerant to Pb and Zn toxicity than is *P. terminalis*, and provide reference ranges for heavy metal concentrations in substrate that will be useful in further studies.

Keywords: accumulation; *Pachysandra terminalis*; polluted urban soil; heavy metal tolerance; *Vinca minor*

Introduction

Certain heavy metals in soil are essential to plants as trace elements, such as iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), and nickel (Ni); they are important constituents of many enzymes and proteins (Hagemeyer, 2004; Naz et al., 2015). Though they are necessary in small quantities, these elements become toxic to plants when they are present at higher concentrations in the soil (Kumar et al., 2013). Lead (Pb) and cadmium (Cd) are not essential for the function of plants (Vesna et al., 2012). Heavy metals inhibit growth and development of plants by disturbing many biochemical and physiological processes (Lai et al., 2012; Maksymiec, 2007). Plants develop a number of unique defense mechanisms and acclimation strategies; these enhance their tolerance to heavy metal stress (Xu et al., 2008).

Heavy metal contamination in soil occurs from both natural and anthropogenic sources in the local ecosystem (Gürcan et al., 2008). While natural occurrence is relatively rare, the number and intensity of anthropogenic sources, such as rubbish tips,

smelter stacks, waste incineration fertilizers, vehicle emissions, agricultural waste, and sewage sludge are important determinants of environmental heavy metal concentrations (Koch and Rotard, 2001). Anthropogenic sources of increased heavy metal content in both terrestrial and aquatic systems are common in urban areas (Kumar et al., 2013).

Rapidly increasing population and unplanned urbanization are challenges to ecological balance (Gürcan et al., 2008). Anthropogenic soil pollution is usually not limited to a single pollutant. Several metals may be present at high concentrations in soil (Marchiol et al., 2004). Cd, Cr, and Ni have been found in contaminated urban soils (Lavado et al., 1998; Naz et al., 2015), as have Cu, Pb, and Zn (Kabala and Singh, 2001; Yoon et al., 2006). Therefore, it is necessary to identify plant species that are resistant to several different kinds of heavy metals, which are able to survive, grow, and reproduce by excluding or accumulating metals present at very high levels in the soil (Franiel and Babczyńska, 2011; Farrag et al., 2012). However, a limited amount of research has been dedicated to describing the behavior of plants capable of accumulating heavy metals in soils contaminated with a number of different metals. Heavy metal uptake is subject to antagonistic, additive, and synergetic effects that heavy metals exert on one another (Marchiol et al., 2004).

Many studies have investigated the tolerance of groundcover plants to contaminated soil, mostly concerning turfgrass species suitable for urban environmental remediation (Dushenkov et al., 1995; Li et al., 2000; Qu et al., 2003), but only a few have focused on groundcover plants grown in an shady urban spaces. Scented geranium (*Pelargonium* sp.) was found to be tolerant to heavy metals via accumulation of the metals (KrishnaRaj et al., 2000; Arshad et al., 2008). In particular, *Pelargonium hortorum* was more tolerant of heavy metals than were other *Pelargonium* species (Orroño et al., 2009). However, there are also little available data on the growth responses of shady groundcover plants to metal stress.

P. terminalis, known as Japanese spurge, is a member of the *Buxaceae* family; it is a frost-hardy evergreen plant widely used for groundcover in Europe and North America owing to its strong shade tolerance and effectiveness in controlling weeds (Lee et al., 2002; Zhu and Beck, 1991; Zhou et al., 2005). *Pachysandra* is an East Asian–North American disjunct genus with two species in eastern Asia, *P. axillaris* and *P. terminalis*, and one species in eastern North America, *P. procumbens* (Zhihua and Jianhua, 2009). This plant displays a high rate of biomass production, tolerates polluted environments, and is useful for soil phytoremediation purposes. It grows best at 24°C to 25°C and can survive in Zone 5 in the United States, where the minimum temperature can drop to -10°C to -20°C (Zhou et al., 2005). The genus *Vinca* in the *Apocynaceae* family comprises seven known species worldwide. Lesser periwinkle (*Vinca minor* L.) is used as an ornamental plant because of its lilac-blue flowers, and is also used as a medicinal plant as an important alkaloid, vincamine, is found in its leaves (Frahanikia et al., 2011). It is a perennial sub-shrub that is indigenous to northern Spain, western France, and central and southern Europe as far as the Caucasus; it has been naturalized in many regions (Mahnaz et al., 2010).

The objects of this pilot study were to determine the heavy metal toxicity threshold, which is the highest permissible content in a substrate used in urban landscaping, and maximum possible accumulation of heavy metals in shoots of *P. terminalis* and *V. minor* grown on substrate containing Cd, Pb, or Zn.

Material and methods

Plant and soil preparation

One-year-old plants (*P. terminalis* and *V. minor*) grown in 12 cm-diameter pots filled with artificial substrates (coco peat:perlite (v/v) = 50:50) were purchased from a local nursery (San-nea Botanical Garden, Chenonan, Chungnam, Korea) on April 15, 2014. All plants used in the experiments were 10-15 cm in height, and had 20-25 fully grown leaves. The plants were placed in a controlled greenhouse with a shade net and allowed to acclimatize for one month prior to transplant. Three replications with ten plants were placed in a randomized complete block design in a greenhouse of KonKuk University, Chungju, located at latitude 35°49'N and longitude 127°08'E. Ten *P. terminalis* and ten *V. minor* plants were transplanted into each of the plastic containers (50 cm wide × 35 long × 8.5 cm deep) on May 17, 2014. The inside of each container was covered with a white plastic sheet to avoid heavy metal leakage. The substrate used for this study was a commercially produced ridging (Shinshin Floricultural materials, Co., Seoul, Korea) composed of 20% peat moss, 15% coco peat, 20% vermiculite, 10% pine bark, 0.5% peat, and 0.5% river sand with pH 6.5. Prior to transplant, each metal was thoroughly mixed with 1 kg of air-dried substrate, and then the mixture was used to fill the containers. The Cd was added at the concentration of 25, 50, or 100 mg·kg⁻¹. The Pb was added at the concentration of 250, 500, and 1,000 mg kg⁻¹. The Zn was added at the concentration of 500, 1,000, or 2,000 mg kg⁻¹. For control treatment, no heavy metal was added to the substrate. The plants were watered at three day intervals (approximately 100 - 200 mL per pot each time) during the experimental period (Fig. 1).



Figure 1. Images of plants grown on substrate containing heavy metals (Cd, Pb, and Zn) in various concentrations.

Growth conditions

Plants were grown in a greenhouse under natural light. During the experimental period, the air temperature and relative humidity were measured by a thermo recorder (SK-1260, SATO, Japan), and photosynthetically active radiation (PAR) was measured by a digital light meter (Extech 401025, EXTECH, USA). Air temperature ranged between 17°C and 35°C, relative humidity between 30% and 70%, and PAR between 0 and 300 $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$.

Measurements

Growth parameters, including plant height, length and width of leaves, number of dead leaves, secondary shoots, number of living leaves, and survival rate of thirty plants per treatment were recorded during the active growth period. Plant height was measured as the distance between the stem base and the shoot apex. Leaf length and width were measured from the 3rd fully expanded leaf above the stem base line. Leaves longer than 0.5 cm in length were counted as the number of leaves. Dead leaves and secondary shoots per plant were counted. Survival rates were calculated by comparing the number of plants in ideal and normal condition versus the number of plants in weakened and dry condition using the following formula: Survival rate = (surviving plants/ total plants) \times 100 (%). After 6 months of growth, the above-ground segments of the plants were harvested; they were washed with water and then rinsed with deionized distilled water to remove foreign objects; they were then oven dried at 60°C for 48 h and ground into powder using a grinder. 2.0 g of dried sample was placed into a 150 mL Erlenmeyer flask with 10 mL concentrated HNO₃ for 24 h, which point 20 mL of ternary solution (HNO₃:H₂SO₄:HClO₄=10:1:4) was added. The sample was initially heated slowly on a 100-200°C hot plate until frothing ceased, and then further heated until the solution became clear and white fumes appeared. Digested plant samples were filtered through Whatman filter paper (No. 42) before metal analysis, and then heavy metal concentrations were determined with an atomic absorption spectrophotometer (ICP, Optima 5300DV, Perkin Elmer, USA). All samples were analyzed in three replicates. Heavy metal concentration was calculated as $C = P \times V \times KTS / m$ (Tian et al., 2013), where C is the heavy metal concentration in the plant (mg kg^{-1}), P is the sample concentration measured by ICP (mg L^{-1}), V is the constant volume of the sample liquid (50 mL), KTS is the divided multiples, and m is the weight of the dried samples (g).

The soil thresholds for heavy metal toxicity and maximum possible accumulation were defined on the basis of a 10% reduction in shoot dry matter yield (Yang et al., 2002).

Statistical analysis

Statistical analyses were done with the SPS program (SPSS Inc., Chicago, IL, USA, Version 18.0), using Duncan's multiple range test (DMRT) to compare means at a significance level of 5%. Single correlation analysis was performed and Pearson's correlation coefficients (r) were reported in order to assess the relationship between heavy metal concentration and growth, as well as that between heavy metal concentration in the substrate and metal accumulation in plants.

Results

Plant growth in relation to heavy metals

The growth and biomass of both *P. terminalis* and *V. minor* were significantly affected by all of the tested heavy metals; Cd affected both to the greatest degree. Cd 25 mg·kg⁻¹ treatment decreased the number of lateral shoots and the number of total leaves of *P. terminalis* by about 43% and 7%, respectively (Table 1). Cd 25 mg·kg⁻¹ treatment decreased the total leaf number of *V. minor* decreased by 33%. There was no significant difference in the lateral shoot number of *V. minor* with either Cd 25 or 50 mg·kg⁻¹ treatments, however, the lateral shoot number decreased by 53% with Cd 100 mg·kg⁻¹ treatment. A 10% reduction in survival rate of *P. terminalis* was caused by both Cd 50 and 100 mg·kg⁻¹ treatments, while the survival rate of *V. minor* decreased only with Cd 100 mg·kg⁻¹ treatment by 10%.

Table 1. Growth and survival rate of *Pachysandra terminalis* and *Vinca minor* as affected by the substrate cadmium (Cd) concentrations during actively growing period (July, 2014).

Species	Added Cd (mg kg ⁻¹)	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Secondary shoots (no. plant ⁻¹)	Total leaves (no. plant ⁻¹)	Dead leaves (no. plant ⁻¹)	Survival rate ^y (%)
<i>P. terminalis</i>	Control	13.3b ^z	4.00a	2.65a	7.50a	28.0a	0.25b	100
	25	12.3ab	3.50a	2.07ab	4.25b	26.0b	0.50ab	100
	50	12.3ab	3.40a	2.00b	3.25b	25.5b	0.65ab	90
	100	10.3b	3.38a	1.80b	3.00b	25.0b	1.80a	90
<i>V. minor</i>	Control	30.7a	2.77a	1.52a	3.75a	49.8a	0.50b	100
	25	16.6b	2.30ab	1.47a	3.75a	33.3b	1.75ab	100
	50	11.5bc	1.85bc	1.13b	3.25a	20.0c	3.25a	100
	100	6.75c	1.65c	1.12b	1.75b	7.00d	3.75a	90

^z Different letters in the same column indicate significant difference according to Duncan's multiple range test at $P \leq 0.05$ (n=10).

^y Survival rate (%) = (surviving plants/total plants) × 100.

Unlike Cd, Pb had no significant effect on the lateral shoot number of *V. minor*, and only affected *P. terminalis* at a concentration of 1,000 mg·kg⁻¹, which resulted in a 59% reduction (Table 2). *P. terminalis* total leaf number was not affected by Pb treatment, while the total leaf number of *V. minor* decreased by 30% and 48% at Pb 500 and 1,000 mg·kg⁻¹, respectively.

There was a significant difference in plant growth responses between the two species following Zn treatment. *V. minor* was more tolerant to Zn than was *P. terminalis*. At Zn 500 mg·kg⁻¹, the secondary shoot number of *P. terminalis* decreased by 53% (Table 3), whereas there was no significant difference in the secondary shoot number of *V. minor* under Zn 500 and 1,000 mg·kg⁻¹ treatments, and there was a 27% decrease at Zn 2,000 mg·kg⁻¹. When treated with Zn 500 mg·kg⁻¹, the total leaf number of *V. minor* actually increased by 54% compared to control plants. With both Zn 1,000 and 2,000 mg·kg⁻¹ treatments, there were no significant differences in total leaf number, dead leaf number, or survival rate of *V. minor*.

Table 2. Growth and survival rate of *Pachysandra terminalis* and *Vinca minor* as affected by the substrate lead (Pb) concentrations during actively growing period (July, 2014).

Species	Added Pb (mg kg ⁻¹)	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Secondary shoots (no. plant ⁻¹)	Total leaves (no. plant ⁻¹)	Dead leaves (no. plant ⁻¹)	Survival rate ^y (%)
<i>P. terminalis</i>	Control	13.3 a ^z	3.50a	2.65a	4.25b	25.5c	0.75a	100
	250	14.5 a	4.00a	2.20ab	7.50a	62.0a	0.75a	100
	500	12.5 a	3.75a	2.00b	5.00b	40.0b	1.00a	100
	1,000	9.75 b	3.25ab	1.75b	1.75c	33.0b	1.25a	100
<i>V. minor</i>	Control	30.7ab	2.77a	1.52a	3.75a	49.8ab	0.50b	100
	250	39.5a	2.67a	1.30ab	4.25a	72.0a	1.50a	100
	500	27.9b	2.42a	1.20ab	3.50a	35.0bc	3.25a	100
	1,000	12.8c	2.17a	1.05b	2.75a	25.8c	3.25a	100

^z Different letters in the same column indicate significant difference according to Duncan's multiple range test at $P \leq 0.05$ (n=10).

^y Survival rate (%) = (surviving plants/total plants) × 100.

Table 3. Growth and survival rate of *Pachysandra terminalis* and *Vinca minor* as affected by the substrate zinc (Zn) concentrations during actively growing period (July, 2014).

Species	Added Zn (mg kg ⁻¹)	Plant height (cm)	Leaf length (cm)	Leaf width (cm)	Secondary shoots (no. plant ⁻¹)	Total leaves (no. plant ⁻¹)	Dead leaves (no. plant ⁻¹)	Survival rate ^y (%)
<i>P. terminalis</i>	Control	13.3 a ^z	3.50a	2.65a	7.50a	25.5a	0.75a	100
	500	14.3 a	3.32a	2.07ab	3.50b	31.8a	0.50a	100
	1,000	11.8 a	3.20a	2.07ab	2.75b	31.3a	0.50a	90
	2,000	11.6 a	2.87a	1.93b	2.50b	28.3a	0.25a	80
<i>V. minor</i>	Control	30.7b	2.77a	1.52a	3.75a	49.8b	0.50a	100
	500	42.5a	2.67a	1.55a	4.00a	76.8a	0.50a	100
	1,000	39.3ab	2.62a	1.52a	3.00a	56.8ab	0.50a	100
	2,000	29.3b	2.37a	1.50ab	2.75ab	47.3b	0.50a	100

^z Different letters in the same column indicate significant difference according to Duncan's multiple range test at $P \leq 0.05$ (n=10).

^y Survival rate (%) = (surviving plants/total plants) × 100.

Correlation coefficient analysis

The Pearson correlation coefficients between the heavy metal concentrations in substrate and the growth of *P. terminalis* showed that, of the three heavy metals tested, substrate Cd concentration was the most positively correlated with shoot Cd concentration ($r=0.98$, $P < 0.01$); substrate Pb and Zn concentrations were also positively correlated with shoot Pb ($r=0.92$, $P < 0.01$) and Zn concentrations ($r = 0.95$, $P < 0.01$) (Table 4). Substrate Cd concentration was negatively correlated with leaf width

($r=0.49$, $P < 0.01$), lateral shoot number ($r=0.47$, $P < 0.01$), and total leaf number ($r=0.48$, $P < 0.01$), while substrate Pb concentration showed a significant negative correlation only with lateral shoot number ($r=0.38$, $P < 0.05$). Substrate Zn concentration was negatively correlated with leaf length ($r=0.35$, $P < 0.05$), leaf width ($r=0.39$, $P < 0.05$), and lateral shoot number ($r=0.56$, $P < 0.01$).

Table 4. The Pearson correlation coefficients between heavy metal concentrations and growth characteristics of *Pachysandra terminalis*.

Metal	Plant height	Leaf length	Leaf width	Secondary shoot no.	Total leaf no.	Dead leaf no.	Shoot metal concentration
Cd	-0.338*	-0.385*	-0.493**	-0.473**	-0.482**	-0.231	0.979**
Pb	-0.025	-0.015	-0.273	-0.376*	-0.122	-0.224	0.917**
Zn	-0.194	-0.345*	-0.393*	-0.561**	-0.221	0.039	0.949**

*,** Significant at $P < 0.05$ or 0.01 , respectively (n=10).

Heavy metal accumulation in shoots in relation to substrate heavy metal concentrations

The Cd $25 \text{ mg} \cdot \text{kg}^{-1}$ caused a reduction in shoot dry weight of about 63% and 30% in *P. terminalis* and *V. minor*, respectively, compared with control plants (Table 5). The Cd $100 \text{ mg} \cdot \text{kg}^{-1}$ treated *P. terminalis* and *V. minor* had 74% and 60% lower shoot dry weights than did their controls, respectively. Both Pb 250 and $500 \text{ mg} \cdot \text{kg}^{-1}$ treatments caused no reduction in shoot biomass of either *P. terminalis* or *V. minor*. The Pb $1,000 \text{ mg} \cdot \text{kg}^{-1}$ treatment caused a reduction in *P. terminalis* dry weight of about 3.7%, whereas no reduction was observed in *V. minor* dry weight. Zn-treated *V. minor* had no reduction in dry weight, whereas for *P. terminalis*, about 11%, 26%, and 52% reductions were recorded under Zn 500, 1,000 and $2,000 \text{ mg} \cdot \text{kg}^{-1}$ treatments, respectively.

The accumulation of Cd in the shoot tissues of both *P. terminalis* and *V. minor* linearly increased when Cd concentration in the substrate was increased to $100 \text{ mg} \cdot \text{kg}^{-1}$. Accumulation of Cd in the shoots was 3- to 4-fold higher in *V. minor* than in *P. terminalis*. With Cd $25 \text{ mg} \cdot \text{kg}^{-1}$ treatment, the shoot Cd concentrations were $2.1 \text{ mg} \cdot \text{kg}^{-1}$ for *P. terminalis* and $5.8 \text{ mg} \cdot \text{kg}^{-1}$ for *V. minor* (Table 5). With Cd $100 \text{ mg} \cdot \text{kg}^{-1}$ treatment, the shoot Cd concentrations were 4.5 and $11.5 \text{ mg} \cdot \text{kg}^{-1}$ in *P. terminalis* and *V. minor*, respectively. The shoot Pb concentrations for both species increased when the concentration of Pb in the substrate increased. The shoot Pb concentration of *P. terminalis* was $1.2 \text{ mg} \cdot \text{kg}^{-1}$ with Pb $250 \text{ mg} \cdot \text{kg}^{-1}$ treatment, almost 2-fold higher than that of control plants ($0.71 \text{ mg} \cdot \text{kg}^{-1}$), however, very little change was seen in the shoot Pb concentration between Pb 250 and $1,000 \text{ mg} \cdot \text{kg}^{-1}$ treatments, with $1.43 \text{ mg} \cdot \text{kg}^{-1}$ being the maximum value at a substrate Pb concentration of $1,000 \text{ mg} \cdot \text{kg}^{-1}$. The shoot Pb concentration of *V. minor* increased 1.6-fold, from 1.25 to $1.96 \text{ mg} \cdot \text{kg}^{-1}$, when Pb concentration in the substrate was increased from 500 to $1,000 \text{ mg} \cdot \text{kg}^{-1}$. The shoot Zn concentration of both species increased with increasing external Zn levels. The shoot Zn concentrations of *P. terminalis* were 0.32, 5.21, 15.3, and $38.1 \text{ mg} \cdot \text{kg}^{-1}$, and those of *V. minor* were 2.12, 6.54, 11.8, and $34.2 \text{ mg} \cdot \text{kg}^{-1}$, when grown in substrate with Zn added at concentrations, 0, 500, 1,000, and $2,000 \text{ mg} \cdot \text{kg}^{-1}$, respectively.

Table 5. Shoot dry weight, shoot metal concentration and reduction of shoot dry weight relative to control plant of *Pachysandra terminalis* and *Vinca minor* as influenced by heavy metals added to substrate at different concentrations.

Species	Heavy metal treatments (mg kg ⁻¹)	Shoot dry weight (g plant ⁻¹)	Shoot metal concentration (mg kg ⁻¹)	Reduction of shoot dry weight relative to control plant (%)
<i>P. terminalis</i>	Cd0	2.7 ^{az}	0.04 ^d	-
	Cd25	1.0 ^b	2.15 ^c	63
	Cd50	0.9 ^b	2.69 ^b	67
	Cd100	0.7 ^c	4.51 ^a	74
	Pb0	2.7 ^{ab}	0.71 ^c	-
	Pb250	2.9 ^a	1.20 ^b	0
	Pb500	2.8 ^{ab}	1.33 ^a	0
	Pb1,000	2.6 ^b	1.43 ^a	3.7
	Zn0	2.7 ^a	0.32 ^d	-
	Zn500	2.4 ^b	5.21 ^c	11
	Zn1,000	2.0 ^c	15.3 ^b	26
	Zn2,000	1.3 ^d	38.1 ^a	52
<i>V. minor</i>	Cd0	1.0 ^a	0.94 ^d	-
	Cd25	1.0 ^a	5.78 ^c	30
	Cd50	0.8 ^b	8.38 ^b	50
	Cd100	0.7 ^b	11.5 ^a	30
	Pb0	1.0 ^b	0.43 ^d	-
	Pb250	1.3 ^a	1.19 ^c	0
	Pb500	1.2 ^a	1.25 ^b	0
	Pb1,000	1.0 ^b	1.96 ^a	0
	Zn0	1.0 ^c	2.12 ^d	-
	Zn500	1.6 ^a	6.54 ^c	0
	Zn1,000	1.3 ^b	11.8 ^b	0
	Zn2,000	1.2 ^b	34.2 ^a	0

^z Different letters in the same column indicate significant difference according to Duncan's multiple range test at $P \leq 0.05$.

Discussion

The results from this pilot study indicate that excessive levels of heavy metals added to substrate significantly affect the growth and development of two groundcover plants commonly used in urban landscapes. The Cd caused the greatest degree of growth inhibition in both species (*Tables 1, 2, and 3*). Some previous studies support this result. The Cd is more toxic than Cu, Pb, and particularly Zn (Kahle, 1993) because of its high solubility in water and its phototoxic effects (Wang et al., 2008). The Cd damages membranes and disrupts membrane electron transport, interacts with nucleic acids, and reduces mitotic activity (Zhang et al., 2009). Also, the Cd is the heavy metal most readily absorbed by plants. The Cd toxicity causes leaf rolls, chlorosis, and reduced plant growth (Zhang et al., 2009).

The Pearson correlation coefficients between the heavy metal levels and the growth of *P. terminalis* shows that shoot numbers and metal accumulation in the shoots are good indicators of toxicity for all three tested metals (*Table 4*). This study aimed to determine the heavy metal toxicity threshold in the substrate that caused a reduction in shoot dry matter of at most 10%; however, the substrate threshold and maximum

possible accumulation in shoots was obtained only for Zn toxicity in *P. terminalis* (Table 5). The substrate Zn threshold was $500 \text{ mg}\cdot\text{kg}^{-1}$ and the maximum possible accumulation of Zn was $5 \text{ mg}\cdot\text{kg}^{-1}$ (Table 5). The lowest Cd concentration applied to the substrate in this study, Cd $25 \text{ mg}\cdot\text{kg}^{-1}$ treatment, caused shoot Cd accumulation of about $2.1 \text{ mg}\cdot\text{kg}^{-1}$ for *P. terminalis* and $5.8 \text{ mg}\cdot\text{kg}^{-1}$ for *V. minor*, which exceed acceptable tissue levels of Cd by between 0.02 and $0.5 \text{ mg}\cdot\text{kg}^{-1}$ (Ward, 1995).

In contrast, the highest Pb concentration in the substrate, Pb $1,000 \text{ mg}\cdot\text{kg}^{-1}$, caused only a 3.7% reduction in biomass of *P. terminalis*, and no significant biomass reduction in *V. minor* was observed. The shoot concentrations of Pb in *V. minor* and *P. terminalis* were 1.4 and $2.0 \text{ mg}\cdot\text{kg}^{-1}$, respectively (Table 5), which are in the range of adequate tissue levels of Pb (between 0.1 and $10 \text{ mg}\cdot\text{kg}^{-1}$) (Bohn et al., 1989). Although plants are mainly sensitive to the presence of Pb (Pahlsson, 1989) because of its inhibitory effects on photosynthesis (Qureshi et al., 2007), a soil Pb concentration of $1,000 \text{ mg}\cdot\text{kg}^{-1}$ did not prove to be the absolute threshold value for hyperaccumulation (Baker, 2000). Pb concentration over $30 \mu\text{g}\cdot\text{g}^{-1}$ dry biomass is toxic to most plant species (Qureshi et al., 2007). These previous results indicate that the two species in the present study can be considered tolerant to Pb.

The results of shoot biomass and shoot Zn accumulation measurements suggest that *V. minor* is more tolerant than is *P. terminalis* to Zn (Table 5). The maximum possible accumulation of Zn, $5 \text{ mg}\cdot\text{kg}^{-1}$, did not exceed the toxicity value, about $100 \text{ mg}\cdot\text{kg}^{-1}$ (Anjum et al., 2015), but was in the range of adequate tissue Zn levels, variously reported as 15 to $20 \text{ mg}\cdot\text{kg}^{-1}$ dry weight (Marschner, 1995), and 10 to $100 \text{ mg}\cdot\text{kg}^{-1}$ dry weight (Frisberg et al., 1986). Although some crops show toxicity symptoms at Zn levels less than $100 \text{ mg}\cdot\text{kg}^{-1}$ dry weight (Chaney, 1997), the concentration of Zn that is toxic to most crops is between 100 and $300 \mu\text{g}\cdot\text{g}^{-1}$ (Marschner, 1995). In the present study, the Zn level added to the substrate was much higher than is normal in soils; it comprised a total fraction of 70 to $400 \text{ mg}\cdot\text{kg}^{-1}$ of the soil (Anjum et al., 2015), indicating that *V. minor* is as a Zn tolerant plant, although it is not a hyperaccumulator. These results provide reference ranges for the heavy metal concentrations in substrate for further studies.

According to a previous report, hydroponics is the most frequent method used to determine the effect of heavy metals as it allows for direct exposure of plants to toxic metals (Zeliha et al., 2011). A recent study, in which 18 populations were cultivated in 18 different growth conditions, such as a soil mine tailing, soils supplemented with Zn, Cd, and Ni salts, and a hydroponic solution with two Zn concentrations, showed a large heterogeneity of responses among populations depending on the substrate used, although culture soils are closer to the conditions in fields than are hydroponics (Escarré et al., 2013). This report also suggests that it is necessary to conduct toxicity testing on different plant species using various substrates.

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