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Physiological Responses and Phytoextraction Potential of *Pinus thunbergii* on Cd-contaminated Soil

Sim-Hee Han[1]*, Du-Hyun Kim[1], Venecio U. Ultra Jr[1,2] and Jae-Cheon Lee[1]

[1]*Department of Forest Resources & Development, Korea Forest Research Institute, Suwon 441-350, Republic of Korea*

[2]*University of Eastern Philippines, Catarman, Northern Samar 6400, Philippines*

Abstract : We investigated physiological responses and phytoextraction ability of *Pinus thunbergii* in cadmium contaminated soil as part of our efforts in identifying plant materials for the restoration and revegetation of forest soil contaminated by heavy metals. Thirty seedlings (ten per treatment) were assigned to three treatments (control, 0.3 and 0.6 mM $CdSO_4$ solution) at first year experiment. At second year, ten seedlings per treatment treated with Cd during the first year experiment were divided by two groups (no Cd-treated and consecutive Cd-treated group). At first experiment, photosynthetic pigment content, and superoxide dismutase (SOD) and glutathione reductase (GR) activities have significantly reduced by Cd application, and the reduction rate was increased much higher as the rate of Cd application increased. On the other hand, thiol and malondialdehyde (MDA) content were significantly increased at the application of 0.6 mM of Cd. At the second year experiment, a general increase in chlorophyll and carotenoid content was observed with Cd treatment while SOD and GR activities showed a relative reduction compared to the control. Similar to the first year measurement, thiol and MDA contents also increased considerably due to Cd treatment. At harvest, dry matter was significantly reduced by Cd treatment especially at the rate of 0.6 mM Cd, but dry yield of *P. thunbergii* treated with 0.3 mM Cd was less affected and it was comparable with the control seedling. Cadmium concentration in seedling tissues increased with increasing Cd application rate while Cd uptake was higher in seedlings supplied with 0.3 mM Cd, which could be ascribed to their high dry matter. Overall, our study has demonstrated the unique physiological response of *P. thunbergii* to Cd-prolonged exposure by showing that the changes in photosynthetic pigment content and antioxidative enzyme activities were dependent on the concentration and duration of treatment. In addition, our results have demonstrated the potential of *P. thunbergii* to withstand up to 0.3 mM Cd (equivalent to cumulative Cd concentration of 134.4 to 268 mg kg^{-1}) without showing growth reduction, hence it might be used for phytoremediation of Cd contaminated areas.

Key words : *cadmium, photosynthetic pigments, physiological response, phytoextraction*, Pinus thunbergii

Introduction

Alongside with industrial development, the associated drawbacks surfaced and threatened environmental sustainability. Example of which is the case of progressive mining activities that created serious problems threatening sustainable development and safe environment. Many of the mining industries lead to environmental hazard especially in abandoned and mined areas abound with mine waste contaminated with high levels of toxic metals. Cadmium is considered as one of the most hazardous to human and ecosystem health due to its high mobility in soils and potential toxicity to biota at low concentrations (Das *et al*., 1997). Although Cd is naturally present in soils at trace amounts, high levels of Cd have been reported in some soil environments. Cadmium is usually associated with several ores of precious mineral which could serves as point sources for Cd contamination in different ecosystems (Rodriguez *et al*., 2009). Widespread Cd contamination of land is also caused by the application of sludge or urban composts, pesticides, fertilizers, emissions from waste incinerators and irrigation with contaminated waste water (Yang *et al*., 2004). Because Cd is readily taken up by roots and translocated into leaves in many plant species, Cd is easily transferred

*Corresponding author
E-mail: simhee02@forest.go.kr

from the soil into the food chain, threatening human and animal health (Järup, 2003).

Current restoration strategies of containing Cd and other heavy metal contaminated mined areas include integrated approaches of landscape engineering and the application of appropriate technologies that would restore the ecological balance of the environment. In most cases, mined areas are devoid of sufficient vegetation that would stabilize the contaminated substrate and prevent these materials from migrating into vulnerable environments, especially the arable agricultural lands devoted for crop production. Consequently, phytoremediation is a potent strategy in restoring ecological health of mined areas because of its capability to stabilize the pollutants (phytostabilization), potentially decontaminate polluted environment and at the same time restore ecological balance by improving soil health and ecosystem.

The success of phytoremediation technology relies greatly on using plant species that are capable of surviving in polluted environment and at the same time accumulate heavy metals on the above ground parts. Several tree species have been identified as potent material for phytoremediation ((Dickinson, 2000; Han *et al*., 2006a) but still there is a need to search for other candidates especially among the indigenous plant resources in a particular eco-climatic zone. Most of the abandoned mining areas are usually prone to erosion because of lack of stable vegetation. In addition, heavy metal availability including Cd in forest ecosystems is also increasing due to deposition of heavy metal containing airborne particulate from air pollution in combination with acid rain occurrence resulting to deterioration of forest trees and ecosystem. Therefore, a thorough understanding of Cd uptake and its effect on growth and development in forest tree species is of major importance to avert heavy metal pollution and forest degradation.

In this study, we tried to assess the potential of *Pinus thunbergii* as a candidate tree species for phytoremediation application in Cd contaminated environment. This tree species is frequently found on the tailing of abandoned mining area, and native to northeastern China, Korea and Japan and a popular landscape tree in Japan and in coastal gardens in northern Europe and the northeastern U.S. It is very tolerant of high winds and salt spray, used to stabilize sand dunes (Tadaki, 1992), and reported to be tolerant of urban conditions which is associated with serious air pollution (Tsukahara *et al*., 1985). To accomplish this goal, we have conducted experiments in order to determine the physiological responses of *P. thunbergii* on elevated Cd concentrations supplied at different rates and period of Cd treatment. Similarly, we also determined the Cd uptake potential of *P. thunbergii* at varying Cd concentration and frequency of application.

Materials and Methods

1. Plant material and treatment application

Pinus thunbergii seeds were collected from the closed zinc-minning area in Goseong, Gyeongnam Province and germinated in a greenhouse. One-year-old seedlings of *P. thunbergii* were transplanted into plastic pots (2L volume) containing river sand. At first year, thirty seedlings (ten per treatment) with uniform standing (average height: 4.3 cm) were selected and subjected with Cd treatments. The first year experiment has three treatments including control without Cd application, and it consisted of control, 0.3 mM Cd (T1), and 0.6 mM Cd application (T2). At second year, ten seedlings per treatment treated with Cd at first year were divided by two groups: non Cd-treated and consecutive Cd-treated group. The second year experiment consisted of T1T0-supplied with 0.3 mM Cd during the first year and no Cd during the second year, T1T1-supplied with 0.3 mM Cd during the first and the second year, T2T0-supplied with 0.6 mM Cd during the first year and no Cd during the second year, and T2T2-supplied 0.6 mM Cd during the first and the second year. Thirty days after transplanting under screen house conditions, Cd was applied weekly over a five-month period from May to September. At each Cd application, approximately 200 mL of 0, 0.3 and 0.6 mM $CdSO_4$ solutions were applied to the seedlings during the first year. On the second year, only seedlings belonging to T1T1 and T2T2 were supplied with 200 mL solution of 0.3 and 0.6 mM $CdSO_4$ while other treatments (T1T0 and T2T0) were supplied with distilled water. The pots were placed in plastic dishes to retain leached nutrients and $CdSO_4$ solution. Pots were randomized in the greenhouse and moved about every two or three weeks throughout a five-month experimental period to minimize positional effects. During the experimental period, daily mean temperature and relative humidity were 23.1±2.1°C and 74.3 ±10.9%, respectively.

2. Pigment analysis

Photosynthetic pigments of *P. thunbergii* were analyzed from 5-month-old (new) needles for the first and second measurement, respectively. Fresh needles (0.1 g) were excised and soaked in 10 mL DMSO (dimethyl sulfoxide) in a glass vial after five months of Cd application (September 2005 and September 2006). The vial was tightly capped and incubated at 70°C for 2h in the dark. The concentration of the extracted pigments (total chlorophyll, chlorophyll a, chlorophyll b, and carotenoid) was calculated based on their absorbance values at 664, 645, and 470 nm according to Lichtenthaler (1987).

3. Antioxidant enzyme activities

Fresh needles (0.1 g) collected on September 2005 and

September 2006 were homogenized under ice-cold condition with 5 mL of 50 mM phosphate buffer (pH 7.0), 10 mM ascorbic acid (AsA) and 1.0% (w/v) polyvinylpyrolidone (Han *et al*., 2006b). The homogenate was centrifuged at 20,000 × g for 30 min, and the supernatant was collected for enzyme assays.

Superoxide dismutase (SOD) was assayed based on the inhibition of reduction of nitro-blue tetrazolium in the presence of xanthine at 530 nm according to the method of Beauchamp and Fridovich (1971). Activity of glutathione reductase (GR) was assayed as described in Carlberg and Mannervik (1985). The assay was carried out in a reaction mixture containing 50 mM phosphate buffer (pH 7.8), 0.1 mM NADPH, 0.5 mM GSSH and 0.1 mL enzyme extract. The change in A_{340} was recorded for 5 min after the addition of enzyme extract.

4. Thiol content

Freshly harvested needles (0.1 g) after the five month duration of Cd treatment were homogenized in 1.5 mL cold buffer containing 5% (w/v) 5-sulfosalicylic acid and 6.3 mM DTPA. The homogenized samples were centrifuged (15,000×g, 10 min) and 250 μL of extract was mixed with 50 μL of Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) in 2.5 mL of 0.1 M sodium phosphate buffer (pH 8) containing 1 mM EDTA. Then, the mixtures were incubated at room temperature for 15 minutes. The absorption was recorded at 412 nm against a blank sample.

5. MDA content

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by thiobarbituric acid reaction as described by Heath and Parker (1968). A crude extract was prepared using fresh needles (0.1 g) homogenized in 5 mL of 62.5 mM phosphate buffer (pH 7.8). The crude extract was mixed with the same volume of 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice-bath. The mixture was centrifuged at 3000 × g for 10 min and the absorbance of the supernatant was monitored at 532 and 600 nm using UV-120 (SHIMADZU, Japan). After subtracting the non-specific absorbance (600 nm), MDA concentration was determined by its molar extinction coefficient (155 mM^{-1} cm^{-1}) and the results expressed as mol MDA g^{-1} FW.

6. Biomass and cadmium determination

At harvest (September 2006), shoots and roots were carefully removed, and then thoroughly rinsed with distilled water twice. The needle, stem and root were partitioned and individually put in paper envelops. Oven dry weights were obtained after oven drying the tissues at 70°C to constant weight.

Dried needle, stem and root (0.5 g each) were ground in a grinding mill (Retsch MM200, Germany). Nitric acid (70%, 15 mL) and hydrogen peroxide (30%, 5 mL) were added to 0.5 g of dried, ground seedling sample in a digestion vessel. Samples were digested using the microwave digestion system, cooled after addition of distilled water, and filtered prior to analysis. Cadmium concentration in the digested tissue was measured by atomic absorption spectrophotometer (AA-6701F, Shimadzu, Tokyo, Japan).

7. Data analysis

The data obtained during the first and the second year was independently subjected to analysis of variance based on complete randomized design at 5% level of significance. There were 3 treatments during the first year and 5 treatments on the second year. SAS System for Windows, Version 8.01 (SAS Institute Inc, 1996) was utilized for the analysis. When significant differences ($P < 0.05$) were indicated, Tukey's Tests (HSD) were performed.

Results

1. Photosynthetic pigments

Photosynthetic pigment in the needles of *P. thunbergii* was affected by Cd treatment applied at different rates and frequency within the two-year period of experimentations (Table 1). During the first measurement on September 2005, a significant reduction on *chlorophyll a* (*Chl a*) was observed in seedlings supplied with 0.6 mM Cd by up to 58% based on the control. Similarly the *chlorophyll b* (*Chl b*) and the total chlorophyll content were reduced significantly with Cd treatment and that higher reduction was observed with increasing Cd dosage. The carotenoid (*Car*) content was also significantly reduced due to Cd application especially at 0.6 mM Cd. The reduction due to 0.6 mM Cd application were up to 60%, 59% and 51% based on the control for *Chl b, total Chl* and *Car*, respectively. Cadmium application at 0.3 mM also resulted to lower photosynthetic pigment content as compared with the control seedlings but such reduction was insignificant. The ratio of *Chl a/Chl b* in seedlings supplied with 0.3 mM Cd was significantly higher compared to the control but not with those seedlings supplied with 0.6 mM Cd. On the other hand, the *Chl a+b/Car* ratio were also reduced with the application of Cd at 0.6 mM.

During the second measurement (September 2006), the *Chl a*, *Chl b*, *Car* but not the total chlorophyll were significantly affected by different Cd treatments. Majority of the seedlings treated with Cd have *Chl a*, *Chl b*

Table 1. Photosynthetic pigment content of *Pinus thunbergii* as affected by different dosage of Cd application[1].

Cd Treatments			*Chl a*	*Chl b*	*Tchl*	*Car*	*Chl a/Chl b*	*Chl a+b/Car*
	Year 1*	Year 2	(ug g^{-1})					
	*September 2005***							
Control	0		1.53±0.19 a	0.41±0.05 a	1.94±0.23 a	0.31±0.04 a	3.79±0.16 b	6.18±0.26 a
T1	0.3 mM		1.37±0.29 a	0.32±0.07 b	1.70±0.07 ab	0.28±0.06 a	4.26±0.16 a	6.12±0.26 a
T2	0.6 mM		0.64±0.03 b	0.16±0.02 c	0.80±0.02 b	0.15±0.01 b	3.99±0.32 ab	5.20±0.27 b
	September 2006							
Control	0	0	0.33±0.01 c	0.10±0.01 ab	0.44±0.02 a	0.08±0.00 c	3.31±0.33 a	5.54±0.36 a
T1T0	0.3 mM	0	0.42±0.01 b	0.11±0.02 ab	0.53±0.02 a	0.09±0.00 b	3.78±0.44 a	5.76±0.43 a
T1T1	0.3 mM	0.3 mM	0.42±0.01 b	0.11±0.02 ab	0.53±0.04 a	0.10±0.00 ab	3.82±0.76 a	5.38±0.58 a
T2T0	0.6 mM	0	0.30±0.00 d	0.08±0.01 b	0.38±0.01 a	0.06±0.00 d	3.65±0.34 a	5.85±0.19 a
T2T2	0.6 mM	0.6 mM	0.51±0.00 a	0.13±0.01 c	0.64±0.02 a	0.11±0.00 a	3.94±0.35 a	5.94±0.18 a

[1]Means within the same column and date of measurement followed by the same letter (s) are not significantly different from each other based on Tukey's HSD test @ 5% level.
*Represent the year of Cd application.
**Date of measurement.

Table 2. Enzyme activities, thiol and MDA content of the needles of *Pinus thunbergii* as affected by different dosage of Cd application[1].

Cd Treatments			*SOD*	*GR*	*Thiol*	*MDA*
	Year 1*	Year 2	unit g^{-1}	nmol g^{-1}	mmol g^{-1}	μmol g^{-1}
	*September 2005***					
Control	0		123±6 a	326±212 a	0.64±0.00 b	7.52±0.11 b
T1	0.3 mM		87±47 a	161±43 a	-	-
T2	0.6 mM		104±89 a	144±57 a	0.7±0.04 a	11.74±0.06 a
	September 2006					
Control	0	0	279±47 a	179±30 a	0.66±0.01 c	8.64±0.05 d
T1T0	0.3 mM	0	207±50 b	232±62 a	0.89±0.08 a	11.65±0.05 a
T1T1	0.3 mM	0.3 mM	176±28 b	180±59 a	0.71±0.00 bc	10.02±0.04 b
T2T0	0.6 mM	0	319±36 a	163±18 a	0.73±0.01 b	9.29±0.06 c
T2T2	0.6 mM	0.6 mM	155±23 b	186±7 a	0.75±0.01 b	8.38±0.01 d

[1]Means within the same column and date of measurement followed by the same letter (s) are not significantly different from each other based on Tukey's HSD test @ 5% level.
*Represent the year of Cd application.
**Date of measurement.

and *Car* contents significantly higher than the control except for seedlings treated with 0.6 mM during the first year of treatment application (T2T0). The pigment content in T1T0, T1T1 and T2T2 increased within the range of 24% to 51% for *Chl a*, 9% to 26% for *Chl b* and 16% to 36% for *Car* over the control seedlings. On the other hand, the pigment content in T2T0 was reduced of up to 11%, 19% and 18% over the control for *Chl a*, *Chl b* and *Car*, respectively. There was no significant difference on total chlorophyll (*Tchl*) content, the ratio of *Chla/Chlb* and the ratio of *Chla+b/Car* among the different treatments. Highest photosynthetic pigment content was observed in seedlings supplied with 0.6 mM Cd for two consecutive years.

2. Antioxidative enzyme activity and thiol and MDA content

The superoxide dismutase (SOD) and glutathione reductase (GR) activities, and the thiol and MDA contents of needles determined on September 2005 and at the end of experiment were presented in Table 2. During the first measurement, the SOD and the GR activities in the needles of *P. thunbergii* exposed to Cd treatment did not gave significant differences with that of the control although Cd treatment reduced the SOD and GR activity of Cd treated seedlings. In contrast, the thiol and MDA content was significantly increased due to the application of 0.6 mM Cd on seedlings.

During the second measurement (September 2006),

the SOD activity showed significant reduction due to Cd treatments as compared to the control except for those in T2T0. Based on the control, a reduction of up to 25%, 37% and 44% in SOD activity was observed in T1T0, T1T1 and T2T2, respectively, while a 14% increase in T2T0 was observed. Although non-significant, majority of the Cd treated seedlings showed a noticeably lower GR content than the control especially at higher Cd concentration or at longer Cd exposure. Similarly, Thiol and the MDA content increased in seedlings treated with Cd solution during the second measurement. The increase of thiol content due to Cd treatment ranged from 7.5% to 36%. Highest increased was observed in seedlings supplied with 0.3 mM Cd during the first year of treatment application. Similarly, the MDA content was significantly affected by Cd treatment. All Cd treated seedlings have higher MDA content than the control except for those seedlings supplied with 0.6 mM Cd for two consecutive years (T2T2). The increase in MDA was up to 35% observed in T1T0-seedlings based on the control.

3. Biomass

Cadmium treatment reduced needle, stem, root and total dry weight of seedlings (Table 2). The greatest significant reduction in dry matter yield was observed in seedlings exposed to high dose of Cd at 0.6 mM (T2T0 and T2T2) with more than 80% decreased based on the control. Seedling supplied with 0.3 mM during the first year only (T1T0) have significantly lower needle, stem and total dry yield compared to those seedlings supplied with 0.3 mM Cd for the two consecutive years (T1T1). Although there was a lower dry yield in T1T1 seedlings compared to the control, their difference was not found to be significant. There was no significant difference in terms of the dry yield shoot/root ratio among the Cd treatments and the control.

4. Cd concentration and uptake

Cadmium concentration in the needle, stem and root of *P. thunbergii* was significantly increased by Cd treatment (Table 3). The Cd concentration in the needles, stem and roots ranged from 45-275 mg kg^{-1}, 70-380 mg kg^{-1}, and 379-536 mg kg^{-1}, respectively. Highest Cd concentration in the tissues were determined on seedlings supplied with 0.6 mM Cd for two consecutive years (T2T2), followed by those seedlings supplied with Cd at 0.6 mM during the first year (T2T0) and these seedlings have more than 4 times Cd concentration on needle and stem compared to those supplied with 0.3 mM Cd (T1T0 and T1T1).

Cadmium uptake in leaves, stem and roots as well as the total seedling uptake were significantly affected with different dosage and frequency of Cd treatment. Highest needle and stem uptake were observed in seedlings exposed to 0.6 mM Cd for two consecutive years while seedlings supplied with lower dose of Cd (T1T0 and T1T1) have significantly higher root Cd uptake compared to T2T0 and

Table 3. Dry matter yield of *Pinus thunbergii* as affected by different dosage of Cd application[1].

Cd treatments			Dry matter yield (mg $seedling^{-1}$)				Shoot/Root ratio
	Year 1*	Year 2	Needles	Stem	Roots	Total	
Control	0	0	3.86±0.74 a	1.48±0.20 a	2.44±0.49 a	7.78±1.22 a	2.23±0.40 a
T1T0	0.3 mM	0	2.35±0.56 b	0.90±0.27 b	1.69±0.38 b	4.94±0.09 b	1.94±0.36 a
T1T1	0.3 mM	0.3 mM	3.50±0.90 a	1.20±0.26 a	2.20±0.33 ab	6.91±1.33 a	2.14±0.41 a
T2T0	0.6 mM	0	0.43±0.21 c	0.23±0.10 c	0.41±0.29 c	1.07±0.58 c	1.85±0.75 a
T2T2	0.6 mM	0.6 mM	0.58±0.22 c	0.25±0.07 c	0.41±0.11 c	1.24±0.40 c	2.01±0.32 a

[1]Means within the same column and date of measurement followed by the same letter (s) are not significantly different from each other based on Tukey's HSD test @ 5% level.
*Represent the year of Cd application.

Table 4. Cadmium concentration and uptake of *Pinus thunbergii* as affected by different dosage of Cd application[1].

Cd treatments			Cd concentration (mg kg^{-1})			Cd uptake (mg $seedling^{-1}$)			
	Year 1*	Year 2	Needle	Stem	Roots	Needle	Stem	Roots	Total
Control	0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
T1T0	0.3 mM	0	53.9±0.5 c	70.3±0.9 d	447.2±2.6 c	135.9±24 b	66.1±21 b	784.2±18 a	986.3±21 a
T1T1	0.3 mM	0.3 mM	45.0±5.6 c	92.2±0.8 c	379.3±3.3 d	154.2±61 b	104.5±22 a	834.7±15 a	1093.3±19 a
T2T0	0.6 mM	0	228.3±1.8 b	311.3±3.4 b	547.8±2.0 a	89.2±52 c	71.5±37 b	224.8±18 b	385.5±27 b
T2T2	0.6 mM	0.6 mM	275.5±10.2 a	379.9±1.4 a	536.4±3.1 b	183.1±43 a	106.3±19 a	236.3±48 b	525.7±10 b

[1]Means within the same column and date of measurement followed by the same letter (s) are not significantly different from each other based on Tukey's HSD test @ 5% level.
*Represent the year of Cd application.

T2T2. On the other hand, total seedling uptake of Cd was higher in T1T0 and T1T1 than those in T2T0 and T2T2.

Discussion

Physiological responses such as photosynthetic pigments, enzyme activities and dry matter yield have been shown to be very sensitive to elevated cadmium in higher plants (Padmaja *et al*., 1990; Skórzyñska-Polit *et al*., 1995; Wu *et al*., 2003; Kim *et al*., 2004). Based on our results, there was a noticeable difference on the measured parameters between the first and the second year of measurement, varying with the degree at which the seedlings were exposed to Cd treatments. During the first year of Cd exposure, *P. thunbergii* responded similarly with other plants toward elevated Cd such as a reduction of photosynthetic pigments and increase in thiol and MDA contents of seedlings (Drazkiewicz, 1994; Abdel-Basset *et al*., 1995). The reduction of *Chl b* is more severe compared to *Chl a* as shown by higher *Chla/b* ratio indicating that for *P. thunbergii* needles, *Chl b* was more sensitive to Cd treatment (Table 1). Conventionally, *chl a* hydrolysis more rapidly compared with *chl b* when seedlings are under stress (Schoch and Brown, 1987; Drazkiewicz, 1994; Abdel-Basset *et al*., 1995) but such phenomenon were not observed on *P. thunbergii*. Abdel-Basset *et al*. (1995) and Ewais (1997) pointed out that heavy metals accumulation would be responsible for the reduction of total chlorophyll. The decrease in chlorophyll concentration may be due to the distortion of chlorophyll ultrastructure, inhibition of synthesis of photosynthetic pigments and enzymes of Calvin cycle (Baryla *et al*., 2001).

In contrast with the first year data, the pigments concentrations in Cd treated seedlings were higher than the control except for T2T0 seedlings. This observation was similar to the response of *Sedum alfredii* subjected to Cd treatment where in the presence of Cd in the nutrient resulted to higher total chlorophyll, chlorophyll a and b content by as much as 32, 30 and 46% of controls, respectively when exposed to 1000 mM Cd. A decline was only found in the presence of 600 mM Cd, and the Cd content in *S. alfredii* leaves was the maximum at this Cd concentration (Zhou and Qui, 2005).

Carotenoids act as light-harvesting pigments and protect chlorophyll and membrane destruction by quenching triplet chlorophyll and removing oxygen from the excited chlorophyll–oxygen complex (Young, 1991). In the case of *P. thunbergii*, the *Car* content in Cd treated seedlings were lower than control during the first year of measurement while prolonged exposure to Cd resulted to general increase especially at 0.3 mM Cd treatment. In this study, carotenoids were less affected than chlorophyll by Cd as shown in a lower *Chla+b/Car* ratio (Table 1). Carotenoids serve as lipid-soluble antioxidants against free radicals and photochemical damage because of conjugated double bonds (Rice-Evans *et al*., 1997). Thus, less effect on carotenoids at lower concentration might represent their supportive role against oxidative stress (Seth *et al*., 2008). Concurrently, our result would imply that for prolonged exposure to Cd stress, the photosynthetic pigments of young needles may not be a prepared parameter in assessing Cd responses of *P. thunbergii*.

Alteration of the oxidant levels in plants is a consequence of the Cd toxicity effects (Foyer and Noctor, 2005) and accumulation of Cd was correlated with the generation of reactive oxygen species (ROS) in sensitive clones of *Holcus lanatus* (Hendry *et al*., 1992). In short term studies, Cd has been shown to elevate lipid peroxidation via ROS formation in plants (Halliwell and Gutteridge, 1989). To scavenge ROS, plants possess a well-organized antioxidative defense system comprising enzymatic and non-enzymatic antioxidants. The cooperative function of these antioxidants plays an important role in scavenging ROS and maintaining the physiological redox status of organisms (Cho and Seo, 2005). Apparently, the exposure of *P. thunbergii* to elevated Cd at long term did not result to increased SOD and GR activities in the needles rather a remarkable decrease was observed especially during the secound year measurement (Table 2). This would imply that in *P. thunbergii*, long term exposure to elevated Cd may not directly increased the production of superoxide such that no activation of the existing enzyme pools or increased expression of genes encoding antioxidative enzymes (Mishra *et al*., 2006).

Plants cope with cellular damage by sequestration of toxic metals away from the cytosol (Hall, 2002). This could be facilitated by complexation with metal-binding peptides, metallothioneins and phytochelatins and may serve to alleviate against Cd toxicity. Thus, the glutathione or phytochelatins might contribute to protect the photosynthesis against Cd toxicity in this plant. In this study, the thiol contents have increased significantly due to Cd treatment (Table 2). This would indicate that *P. thunbergii* has the capacity to tolerate high and prolonged exposure to Cd by synthesizing thiol compounds capable of binding with Cd. Similarly, some authors indicated a direct relationship of higher degrees of metal exposure to an increase in thiol productions and an accumulation of the longer chains (Tukendorf and Rauser, 1990, Lima *et al*. 2006).

The content of malondialdehyde (MDA) was generally increased in the presence of cadmium stress especially at 0.3 mM Cd treatments. However, continued Cd treatment resulted to significant reduction of MDA as com-

pared to those plants treated only for 1 year and that the MDA concentration was generally lower in plants supplied with 0.6 mM Cd than those supplied with 0.3 mM Cd. MDA is a product of lipid peroxidation and an indicator of free radical production and tissue damage. It seems that the tissue damage by free radicals is alleviated by some unknown direct and indirect enzymatic pathways and detoxifying action of carotenoids (Abad and Khara, 2007) during prolonged exposure to elevated Cd concentration.

The ultimate consequence of long term Cd stress in plants could be assessed on the dry matter yield of plants. Our results have shown that *P. thunbergii* could relatively tolerate Cd concentration up to 0.3 mM supplied for up to 2 consecutive years before showing significant reduction in dry matter yield. Increasing the Cd treatment to 0.6 mM Cd abruptly during the first year and continuously during the second year resulted to significant reduction in plant growth as evident in low dry yield. On the other hand, Cd accumulation on plant tissues have generally increased with increasing concentration and that the concentration were in the order of root > stem > needle. This concentration pattern were similar to other plants which is determined by several factors such as the binding of Cd to extracellular matrix (Horst, 1995), complexing inside the cell (Cobbett *et al*., 1998) and on the transport efficiency (Marchiol *et al*., 1996). Additionally, the increased translocation of Cd to shoots with increasing Cd concentration on the growth media may result from the increased transpiration rate and thiol content in plants. Ding *et al*. (1994) reported that the Cd uptake was proportional to the increase of the thiol group content.

The needle Cd uptake by *P. thunbergii* was higher in 0.3 mM Cd than 0.6 mM treatments while higher stem Cd uptake resulted due to prolonged Cd treatments. In contrast, the root uptake and the total plant uptake were higher in seedling supplied with 0.3 mM than those with 0.6 mM Cd. These results imply that the Cd accumulation pattern of *P. thunbergii* is a function of doses, exposure time and the degree at which plants would be able to adapt to Cd stress at a particular growing condition. It appeared that lower Cd concentrations (T1T0 and T1T1) have not disrupted much of the physiological system and transpiration of *P. thunbergii* as compared to the much higher Cd application. It could also be noted that at lower Cd stress, plants could be able to counteract Cd stress by immobilizing them in the root system through cell wall and extracellular carbohydrates (Wagner, 1993). In contrast, Cd concentration at 0.6 mM showed to be toxic to *P. thunbergii* which have resulted in disrupted physiological system, impaired photosynthetic functions and resulted to poor growth and total Cd uptake.

Overall, our study has demonstrated the unique physiologic response of *P. thunbergii* to prolonged exposure to Cd treatment by showing that the change in photosynthetic pigment content and activity of antioxidant enzymes were dependent on the duration of treatment. The results from this experiment suggest that the antioxidant system, besides its function in detoxification, can also be a sensitive target of Cd toxicity in plants. Specifically, Cd-prolonged exposure of *P. thunbergii* enhanced photosynthetic pigment and thiol content that could have been responsible for tolerance to moderate levels of Cd. In addition, our results have demonstrated the potential of *P. thunbergii* to withstand up to 0.3 mM Cd (equivalent to cumulative Cd concentration of 134.4 to 268 mg kg^{-1}) without showing growth reduction, hence it might be used for phytoremediation of Cd contaminated areas.

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