CHAPTER 3
MATERIALS AND METHODS

Materials

Chemicals

nitric acid (HNO_3), GR, 65%, E Merck, Germany (750 ml)
lead nitrate Pb $(NO_3)_2$, GR, 99%, UNIVAR, Australia (50 g)
lead nitrate Pb $(NO_3)_2$, Standard solution, BDH, England (100 ml)
formaldehyde solution, 34-38% ww of CH_2O, The Thai Government Pharmaceutical Organization, Bangkok (100 ml)
hydrogen peroxide (H_2O_2), GR, 30%, CARLO ERBA, Rey (200 ml)
hydrofluoric acid (HF), GR, 40%, Riedel, Dehain (100 ml)
deionized water and distilled water (10 l)

Other items

pure sand from CMU Biology store (40 kg)
urea fertilizer (CO $(NH_2)_2$) (1.5 kg) composition = nitrogen 46%, hydrogen 7%, carbon 20%, oxygen 27%

Apparatus

Atomic absorption/ flame emission spectrophotometer (AAS) Shimadzu AA-680
Analytical balance Mettler type BB3 and Mettler Toledo type Dragon 303, Switzeland
Milestone microwave digestion system (ETHOS PLUS with MPR-300/125 medium pressure rotor)
Drying oven, Scientific Series 9000
SZ, ST Olympus microscope, Japan
Digital lux meter 93421 BEHA, Germany.
Whatman filter papers, 90 mm diameter (2 boxes)
120 plastic petri dishes, 9 cm diameter
sieve, 1 mm, Hubbard Scientific Company
15 modular trays (3"x 4"x 5") one cell size
70 plastic pots and dishes (15 cm diameter); 180 plastic pots and dishes (10 cm diameter)

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Plant species selection

Seven plant species, including five herbaceous species and two woody species, representing seven families were used in this research (Tables 9, 10; Figure 4 and Appendix 1).

Table 9. Plant species tested

Scientific name	Family	Habit	Origin	Common name	Source
Eleusine indica (L.) Gaertn.	Gramineae	weed herb	native	goose grass	Radanachaless and Maxwell, 1994
Euphorbia heterophylla L.	Euphorbiaceae	weed herb	native	euphorbia	"
Brassica rapa L. cv. Chinese cabbage	Cruciferae	cultivated crop herb	introduced	Chinese cabbage	www.Floridata.com. htm. 2003
Pisum sativum L.	Leguminosae, Papilionoideae	cultivated crop herb	introduced	garden pea	"
Helianthus annuus L.	Compositae	cultivated crop herb	introduced	sunflower	"
Lagerstroemia speciosa (L.) Pers. var. *speciosa*	Lythraceae	ornamental tree	native	queen's crape myrtle	Menninger, 1962
Shorea roxburghii G. Don	Dipterocarpaceae	deciduous tree	native	pah yawm (Thai name)	www.flowersandherbs.cscoms.com/2000

Species selection criteria

1. The plant species studied represented three plant types: herbaceous weeds, herbaceous cultivated crops, and trees.
2. One of the main lead contaminated sources is leaded gasoline. Fishbein (1978) noted that plants grown near roadsides were ten or more times richer in lead than those grown further away. Martin and Coughtrey (1982) suggested that roadside plants should be investigated to assess lead pollution risk. Due to this fact, two common roadsides weed species, *viz. Eleusine indica* and *Euphorbia heterophylla* were selected for this study.
3. The U. S. EPA, OECD, and U. S. FDA recommend some cultivated plant species for phytotoxicity tests. *Brassica rapa* L. cv. Chinese cabbage is a recommended species

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and *Pisum sativum* and *Helianthus annuus* are common phytotoxicity test species (Fletcher, 1991; Weatherford *et al.*, 1997; Wierzbicka, 1999).

4. I selected two tree species, *viz. Lagerstroemia speciosa* and *Shorea roxburghii* which have never been tested for phytotoxicity before. Although there are few studies on the phytotoxic effects on tree species, these species should be tested for species selection for revegatation in contaminated areas. Perennial trees are more suitable for revegatation rather than annual or perennial herbaceous species.

Methods

This research consisted of four parts: (1) seed collection and preparation, (2) seed germination tests, (3) seedling growth tests, and (4) lead analysis.

Seed collection and preparation

About 1000 seeds of each species tested were collected from Chiang Mai University or purchased from a seed company (Table 10, Figure 4, and Appendix 1).

Table 10. Seed collection data

Species	Collection date	Collection method	Number of parent plants	Parent plant height (cm)
Eleusine indica	1 July 2003	from parent plants	50	30-45
Euphorbia heterophylla	15 April 2003	from parent plants	70	50-80
Brassica rapa	1 July 2003	purchased from seed company	-	na
Pisum sativum	20 June 2003	purchased from seed company	-	na
Helianthus annuus	25 September 2003	purchased from seed company	-	na
Lagerstroemia speciosa var. *speciosa*	15 June 2003	from parent plant	1	400
Shorea roxburghii	25 June 2003	collected from the ground	1	1500

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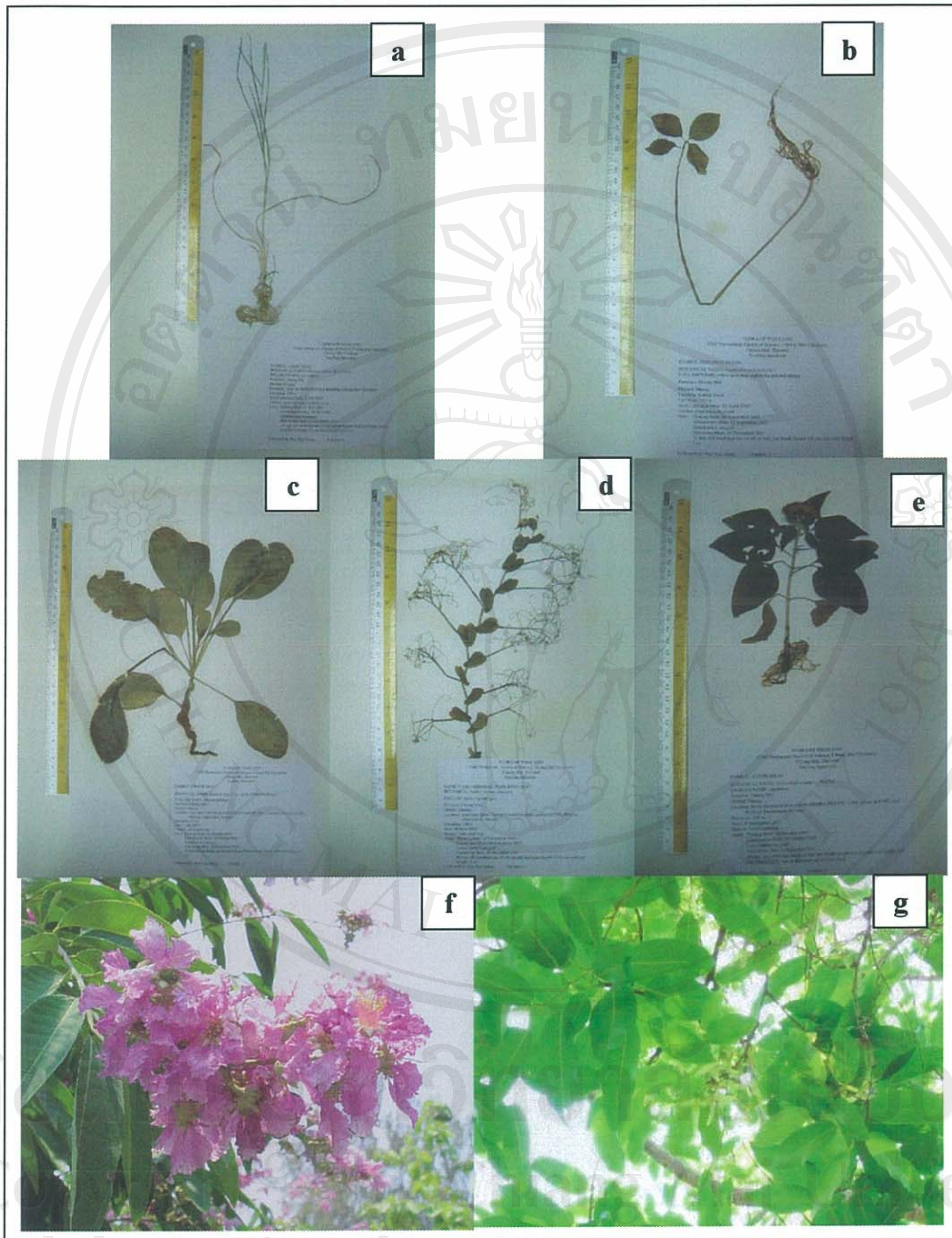

Figure 4. Plants species tested: a-- *Eleusine indica*, b-- *Euphorbia heterophylla*, c-- *Brassica rapa*, d-- *Pisum sativum*, e-- *Helianthus annuus*, f-- *Lagerstroemia speciosa* var. *speciosa* , g-- *Shorea roxburghii* (27 April 2004).

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After collection, seeds of *Eleusine indica* and *Euphorbia heterophylla* were dried and cleaned. The seed coats of *Lagerstroemia speciosa* were cut to make small holes to break down seed dormancy. The five fruiting calyx wings of *Shorea roxburghii* were cut off. Seeds were then immersed in 3% formaldehyde for 5 minutes to prevent fungal growth and washed five times with distilled water to remove the formaldehyde. Good seeds were selected by flotation test, in which, the seeds were soaked in water for about 5 minutes. Good seeds sank while rotten seeds floated. Only good seeds were soaked in water for 12 hours to stimulate germination.

Seed germination test

Seed germination tests were conducted in the Environmental Science Program Laboratory, Faculty of Science, Chiang Mai University during August to October 2003. There were 6 plant species used in this experiment; *Eleusine indica*, *Euphorbia heterophylla*, *Brassica rapa*, *Pisum sativum*, *Helianthus annuus* and *Lagerstroemia speciosa* (Figure 5a). Tests on *Shorea roxburghii* could not be done because the seeds did not germinate, after 2 months which was beyond the germination test period. Seed viability of this species is quite short and the seed have to germinate directly after falling to the ground.

Voucher specimens were prepared and have been deposited in the CMU Herbarium. These specimens include various growth stages of control plants. The data concerning each species is included in Appendix 1.

Experimental procedure

The soaked seeds were placed on filter paper in 9 cm diameter plastic petri dishes. Fifteen seeds were placed in each dish on the surface of the filter paper and 10 ml analytical grade $Pb(NO_3)_2$ aqueous solution was added. The petri dishes were covered and placed in the laboratory. The $Pb(NO_3)_2$ concentrations used in this experiment were 0, 100, 250, 500, and 1000 μg/ml. This follows the method of Xiong (1998). Distilled water was used for the controls and dilutant. The experimental design was a completely randomized block (CRB) method with five treatments and four

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replications. Esposure lasted 120 hours (5 days) for the herbaceous species (Mishra and Choudhuri, 1998). For the tree species (*Lagerstroemia speciosa*), exposure duration was 336 hours (14 days) because the seed germinated 11 days after sowing. So exposure time for this species had to extend after 3 days germination to study their early growth. The experimental conditions are shown in Table 11.

Table 11. Germination test conditions

Temperature	25 ± 3^0 C
Light intensity (lux)	250
Light source	natural light
Container	120 9 cm diameter petri dishes

Measurement

After the exposure period, the percentage of germination was done. The appearance of a stub of a hypocotyl was considered as a germinated seed. The shoot (epicotyl and hypocotyl) and root lengths were measured under a microscope (40 X).

Data analysis

The germination percentage was calculated from the following formula:

$$\text{Percent of germination} = \frac{\text{number of seeds germinated}}{\text{total number of seeds sowed}} \times 100$$

The percent of difference from control (%DFC) for germination was calculated from the following formula (Mishra and Choudhuri, 1999):

$$\% \text{ DFC} = \frac{\% \text{ germination of control} - \% \text{ germination of treatment}}{\% \text{ germination of control}} \times 100$$

The phytotoxicity percentage for shoot and root length elongation were calculated by the following formula (Mishra and Choudhuri, 1999):

$$\% \text{ phytotoxicity} = \frac{\text{shoot or root length of control} - \text{shoot or root length of treatment}}{\text{shoot or root length of control}} \times 100$$

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The measured data and transformed data were subjected to a one-way ANOVA test for completely randomized block design (CRD). The test was carried out using the Statistical Package for the Social Sciences (SPSS) version 10.

Seedling growth test

The seedling growth test was conducted in a nursery in the Department of Biology, Faculty of Science, Chiang Mai University during July to December 2003. All 7 plant species were tested in this experiment (Figure 4), *viz. Eleusine indica*, *Euphorbia heterophylla*, *Brassica rapa*, *Pisum sativum*, *Helianthus annuus*, *Lagerstroemia speciosa* var. *speciosa* and *Shorea roxburghii.*

Experimental procedure

1. Growth media selection

Pure sand, sieved through 1 mm mesh, was used as the growth medium. Hydroponics, sand culture, and soil culture are usually used in growth tests. Soil culture most closely resembles natural condition, but it can be very heterogeneous even in a single batch of soil (Kohl and Losch, 1999). Hydroponic systems provide good reproducibility, accessibility for root observations and harvesting, however, the iron chelator EDTA has a high affinity for some metals and the interaction between EDTA and these metals decreases toxicity (Kohl and Losch, 1999). Sand culture provides a compromise between soil cultures and hydroponics with replicable growing condition, ease of nutrient supplementation due to very low ion content, and better root harvesting than in soil culture (Kohl and Losch, 1999).

2. Seed Sowing

The soaked seeds were sown in modular germination trays filled with pure sand (Figure 5b). These germination trays were placed on benches in the nursery and protected from rain and seed predators. These trays were watered daily until the seeds germinated.

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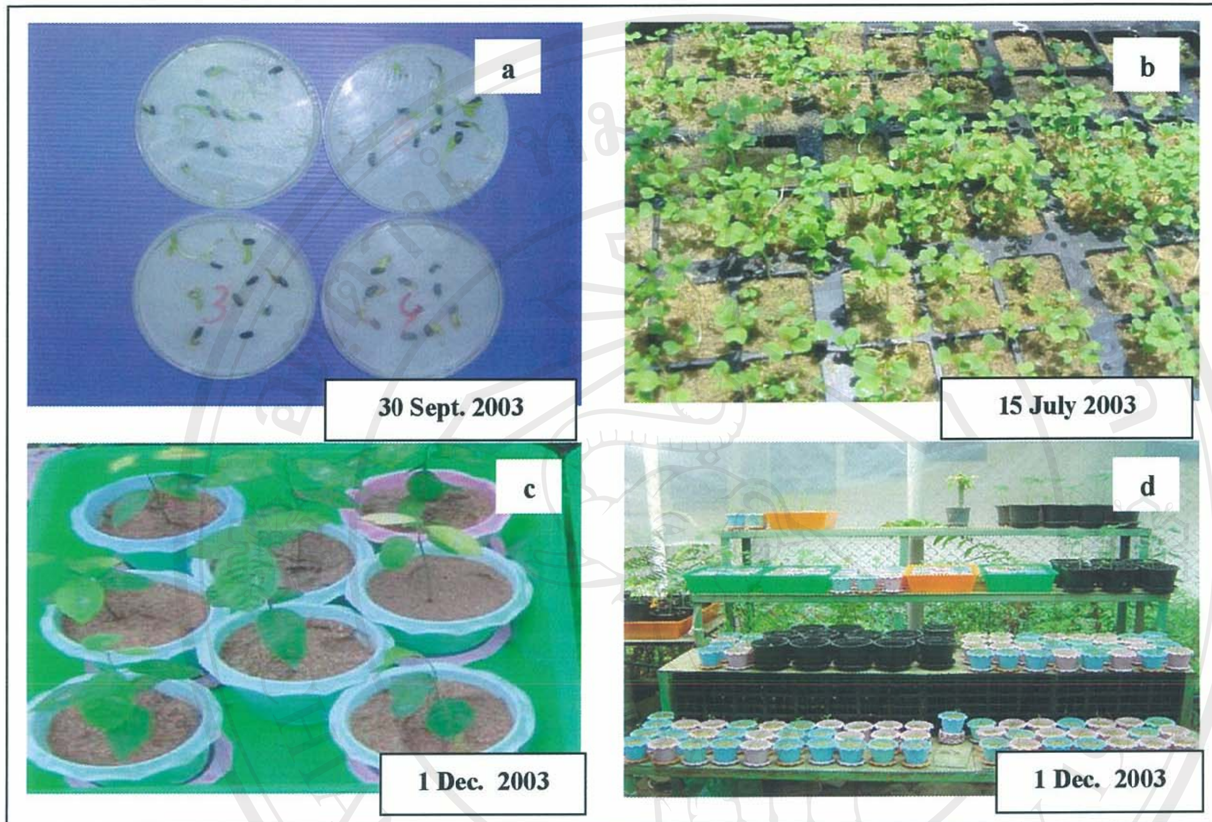

Figure 5. Experimental procedure: a--seed germination tests (*Helianthus annuus*, after 120 hours of lead exposure), b--seed germination trays (*Brassica rapa*, 5 days after germination), c--seedlings in pots (*Shorea roxburghii*, 139 days after germination),

d-- seedlings in a nursery at the Biology Department.

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After hypocotyl, and radicle emergence, seedlings were transplanted into round plastic pots of 15 cm and 10 cm in diameter filled with pure sand (Figure 5c, d). The 15 cm pots were filled with 450 g of dry sand and the 10 cm pots with 350 g. Two g of urea fertilizer was added to each pot and 3 plants were put in each pot. These pots were watered daily and urea was added based on their development (*i.e.* chlorosis) until treatment with lead.

Fertilizer application affects metal concentration in growth media and uptake by plants because some chemicals contained in these nutrients can react with metal ions especially sulphates compounds for lead and copper (Kohl and Losch, 1999). For nitrogen fertilizer, urea fertilizer ($CO(NH_2)_2$), which consist of N, C, H, O elements only should be used rather than ammonium sulphate fertilizer $(NH_4)_2SO_4$.

3. Lead treatment

When the plants reached a height of 4-16 cm depending on the species tested, one seedling was retained in each pot for the lead test and the others culled and some were made voucher specimens (Appendix 1). The experimental design was a randomized complete block (RCB) method with four treatments and four replications. Lead concentrations tested were calculated based on sand weight in the pot and the $Pb(NO_3)_2$ powder was dissolved with 20 ml of distilled water for four replications for each treatment as shown in Table 12. Later 5 ml of aqueous solution with different Pb^{2+} concentrations were added each pot. The lead concentrations in the growth medium were 0, 100, 250 and 500 μg/g for the control and the treatments respectively. Initial plant growth were measured one day before adding lead solutions. During the treatment period, excess water leached out from pots was kept in plastic dish placed below each pot and return to the pot.

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Table 12. $Pb(NO_3)_2$ spiked amount per pot (mg)

Pb treatment (µg/g)	450 g sand/pot	350 g sand/pot
0	0	0
100	72	56
250	180	139
500	360	280

4. Exposure duration

It is important to determine exposure duration for phytoxicity tests. Swanson *et al.* (1991) noted that this duration varied between 1 hour to two months depend on test plant species and endpoints of the tests, *viz.* seed germination, seedling growth, photosynthesis, *etc.* Exposure time of seedling growth tests for herbaceous crops and herbaceous weeds lasted 4 and 5 weeks, respectively (Somashekar and Siddaramaiah, 1991; Tarun *et al.*, 2002). The time for tree species was not recorded. So exposure duration for trees was 8 weeks. The lead toxicity symptoms observed in weekly measurements of trees especially *Shorea roxburghii* were not clear like herbaceous plants. So the exposure time had to extend until 8 weeks, the maximum time for phytotoxicity tests. The experimental conditions are shown in Table 13.

Table 13. Seedling growth test conditions

Temperature	27 ± 4°C
Light intensity(lux)	2000
Light source	natural light
Container	70 15 cm diameter pots 180 10 cm diameter pots

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Measurements

Initial measurements of shoot length, leaf number, leaf blade length, and leaf blade width on both sides of the midrib at the widest point of the lamina were taken one day before adding lead. All parameters were then measured weekly until harvesting

time. In leaf width measurement, three mature leaves were sampled from each plant and measured. The roots were carefully washed with tap water to get rid of the sand, and then washed with distilled water. The plants were separated into roots and shoots (aerial plant parts). The wet weights were determined, individually bagged, labeled, and dried in an oven at 85° C for 2 days to obtain dry biomass. Then the biomass (dry weight) was measured.

Data analysis

Absolute fluctuating asymmetry (FA) was calculated using the following formula (Palmer and Strobeck, 1986):

$$FA = \frac{(R-L)}{Size}\text{; where size} = \frac{(R+L)}{2}$$

Where: R = width of right half of leaf blade

L = width of left half of leaf blade

The relative growth rate (RGR) for height was calculated using the following formula:

$$RGR = \frac{H_1 - H_0}{H_0} \times 100$$

Where: H_1 = shoot height at harvest time

H_0 = shoot height at initial time

Biomass dry weight percent was calculated using the following formula:

$$\%\text{ of dry weight} = \frac{\text{g of dry sample}}{\text{g of wet sample}} \times 100$$

Relative yield was calculated using the following formula:

$$\text{Relative yield} = \frac{\text{yield of treatment}}{\text{yield of control}}$$

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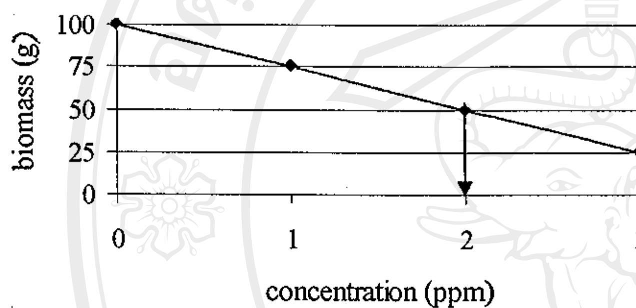
Percent inhibition was calculated using the following formula (El-Khatib, 2003):

$$\text{Percent inhibition (\%)} = 100 - \left(\left\{ \frac{\text{treatment}}{\text{control}} \right\} \times 100 \right)$$

Effective concentration for 50 % inhibition in growth (EC_{50}) were determined using the following graph (Shaw and Chadwick, 1998):



$EC_{50} = 2$ ppm

Figure 6. EC_{50} determination.

Statistical analysis was done using SPSS version 10. The effects of treatments on shoot length, biomass, leaf number, and FA were tested using one-way ANOVA and then Tukey *post hoc* comparisons were done to investigate treatment differences. Paired t-tests were conducted for testing the leaf blade side deformation. Repeated measures ANOVA were done on shoot length and leaf number to investigate the lead treatments effects over time.

Lead analysis

Lead analysis was conducted in a laboratory in the Chemistry Department, Faculty of Science, Chiang Mai University during January 2004.

According to the results of the germination and growth tests of all plants tested, *Euphorbia heterophylla*, *Brassica rapa*, and *Shorea roxburghii* were selected to test lead translocation in plant tissues. These species were selected because:

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1. One species from each plant group, *viz*. herbaceous crop, weed, and tree was selected for analysis, and
2. Based on inhibition percent and relative yield of all species tested, the most sensitive, medium sensitive, and less sensitive species were selected for lead analysis.

Sample preparation

The dried samples were reduced in size and grounded by using a grinder. Then 0.5 g of ground dried samples were weighed, bagged, labeled, and stored until analysis.

Microwave digestion

A closed vessel microwave system with a medium pressure rotor was used in this study. For each plant sample 0.5 g was placed in a Teflon vessel and digested with 8 ml of 65% HNO_3, 2 ml of 30% H_2O_2, and 1 ml of 40% HF in microwave digestion system for 15 minutes. Digestion conditions for the microwave system is given in Table 14. In order to validate the method, 100 ppm of lead solution spiked to each sample including controls and treatments.

Table 14. Operating condition in a microwave digestion system

	Step 1	**Step 2**
Temperature	180^0 C	180^0C
Time	5 minutes	10 minutes
Power	up to 1000 Watts	up to 1000 Watts
Pressure	300 psi	300 psi

Lead determination

An air- acetylene flame atomic absorption spectrometer (FAAS) was used for lead determination. Preparation of a standard solution was done by diluting a stock solution to the working solution in 50 ml volumetric flasks. Measurements were made on a flame AAS machine. Measuring conditions are given in Table 15.

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Table 15. Measuring conditions for AAS

Current	10 mA/ 0mA ; 8 mA/300 mA
Wave length	283.3 nm
Slit width	0.5 nm
Lighting mode	BGC-D2 ; BGC-SR
Burner height	7 mm
Fuel gas flow	2.0 l/ min
Type of oxidant	air

Data analysis

The translocation factor (TF) (Mattina *et al.*, 2003), transfer coefficient (K) (Kloke *et al.*, 1994), and coefficient of variation (CV) were calculated by using following formulae:

$$\text{Translocation factor (TF)} = \frac{\text{concentration in shoots (aerial plant parts)}}{\text{concentration in roots}}$$

$$\text{Transfer coefficient (K)} = \frac{\text{concentration in shoots (aerial plant parts)}}{\text{concentration in soil}}$$

$$\text{Coefficient of variation (\%)} = \frac{S}{X} \times 100$$

The statistical analyses were done using SPSS version 10. The effects of treatment on lead content in different plant tissues were tested using one-way ANOVA and then Tukey *post hoc* comparisons were done to investigate treatment differences. The correlation between lead content, lead treatments in the growth medium, and plant tissues as well as interaction between two factors was tested using multivariate analysis. The correlation between lead content in different plant parts and biomass weight of different plant parts was tested using correlation analysis.

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