Studies on lead phytoremediation by tropical pasture grasses and assessment of contaminated pasture products through animal

（暖地型牧草種による鉛ファイトレメディエーションと家畜を通じての汚染された草地生産物の評価に関する研究）

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CHAPTER 1

General Introduction

Heavy metal pollution of soils is one of the most important environmental problems throughout the world. Anthropogenic activities are believed to be the major causes of environmental pollution although some contamination is derived from natural geological sources. Soil may become contaminated by the accumulation of heavy metals and metalloids through emissions from the rapidly expanding industrial areas, mine tailings, disposal of high metal wastes, leaded gasoline and paints, land application of fertilizers, animal manures, sewage sludge, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals, and atmospheric deposition (Khan *et al*. 2008; Zhang *et al*. 2010). Accumulation of toxic heavy metals such as lead (Pb), arsenic (As), cadmium (Cd), copper (Cu), and zinc (Zn) in agricultural soils not only has detrimental effects on the ecosystem functioning but also poses potential health risks due to transfer of these contaminants into the food chain (soil-plant-human or soil-plant-animal-human) (Kabata-Pendias 1992; Giller *et al.* 1998). Trace amount of some heavy metals are required by living organisms, however any excess amount of these metals can be detrimental to the organisms (Berti and Jacobs 1996). It may be direct ingestion or contact with contaminated soil, drinking of contaminated ground water, reduction in food quality via phytotoxicity, reduction in land usability for agricultural production causing food insecurity, and land tenure problems (McLaughlin *et al*. 2000a; McLaughlin *et al*. 2000b; Ling *et al*. 2007). Foodborne diseases and hazards are significant in all parts of the world, and the reported incidences of diseases have increased over last two decades (FAO & WHO 2002a). Food safety and quality are as important as food security and quantity in human and animal feeding.

Myanmar is endowed with mineral resources such as copper, gold, lead, zinc, silver, tin and nickel and so on. The large lead and zinc concentrates mining operations are Namtu-Bawdwin, Yadanar Theingi and Bawsaing mines in the Shan State of northern Myanmar. In fiscal year 1992, the mine output of lead and zinc increased considerably. Although there has not been reported heavy metals soil contamination data in Myanmar, industrial and agricultural development has been largely responsible for pollution of the environment with toxic metals.

Lead is widely accepted as a major environmental threat. Lead with atomic number 82, atomic weight 207.19, and a specific gravity of 11.34, is a bluish or silvery-grey metal with a melting point of 327.5°C and a boiling point at atmospheric pressure of 1740°C. It has four naturally occurring isotopes with atomic weights 208, 206, 207 and 204 (in decreasing order of abundance). It is an extremely difficult soil contaminant to remediate because it is a soft Lewis acid that forms strong bonds to both organic and inorganic ligands in soil (Cunningham and Berti 2000). Lead in soil may be present in many different forms, in different oxidation states, and associated with different complexation states on soil surfaces. Divalent Pb is often complexed with organic matter, adsorbed onto cation exchange sites on the soil surfaces, or precipitated as relatively insoluble salts (Cunningham *et al*. 1995). The various forms present in soils have different solubilities and bioavailabilities, and each presents a unique environmental risk.

Techniques are imperatively needed to reduce the level of toxic metals in contaminated soils. Conventional methods used for the remediation of metal contaminated soils include soil washing, land filling, chemical treatments and electrokinetics (Salt *et al.* 1995; Glass 1999; Kumpiene *et al*. 2008). However, remediation of heavy metal contaminated soils with conventional methods is prohibitively expensive (Salt *et al*. 1995). It may cost from $10 to 1000 per cubic meter. Therefore, it is needed to approach the environmental friendly and cost effective techniques.

Phytoremediation is a technique that uses various plant species to facilitate soil or water reclamation. The ideal plant characteristics for phytoremediation should have the hyperaccumulation and tolerance of heavy metals, the fast growth and high biomass, a well-developed root system, easy for cultivation and harvesting, and an extensive geographical distribution. Many species of plants have been successful in absorbing contaminants such as lead, cadmium, chromium, arsenic and various radionuclides.

There are five categories in the phytoremediation technique:

(1) Phytodegradation: the use of plants and associated microorganisms for the breakdown of metal contaminants by plant enzymes following uptake from the soil (EPA 2000)

(2) Phytostabilization: the restriction of contaminant mobility and bioavailability in soil

(3) Rhizofilteration: the use of plant roots for the remediation of waste water by aquatic or land plants

(4) Phytovolatilization: the use of plants to extract soil contaminants and then transform them into volatile substances out to the atmosphere

(5) Phytoextraction: the use of plants to translocate metal contaminants from the soil to the ground surface via the root system of plants (Brooks 1998)

Phytostabilization and phytoextraction are two common phytoremediation techniques in treating metal-contaminated soils, for stabilizing toxic soils, and the removal of toxic metals from the soils, respectively. Soil amendments should be added to aid stabilizing soils, and to enhance metal uptake accordingly. Phytoremediation is on average tenfold cheaper than conventional remediation methods (Glass 1999) and it may also become a technology of choice for remediation project in developing countries because it is cost-efficient and easy to implement.

Selection of appropriate plant species would be very important to ensure a self-sustainable vegetation cover. Mostly seeds from local regions are preferred, because it is easier for the plant to adapt to the environment. The root zone is of special interest in phytoremediation to absorb contaminants and store or metabolize it inside the plant tissue. Aprill and Sims (1990) showed that grass roots have the maximum root surface area compared with other plant types and may penetrate the soil to the depth of up to 3 meter. Although grasses are commonly used for the vegetation of contaminated sites, little information is available regarding the tolerance of many of these species to lead. *Brachiaria decumbens* cv. Basilisk (signalgrass), one of the more commonly sown tropical pasture species, is widespread throughout tropical America, south-eastern Asia and the Pacific, and is well adapted to highly acidic soils (Wenzl *et al*. 2001). *Paspalum atratum* Swallen (atratum) is a perennial tussock grass and used as a long-term pasture.

Pennisetum purpureum Schumach (napiergrass) is a high-stalk perennial grass, widely cultivated as forage grasses in south-eastern Asia. These grasses possess the abilities, such as rapid growth rate, large biomass, high resistance to adverse conditions, abundant seeds, ease of cultivation and repeated cropping (Boonman 1993).

Agronomical practices like pH adjustment, addition of chelators and fertilizers are developed to enhance remediation. Heavy metal solubility in soils is mainly controlled by the soil reaction (pH), the amount and kind of sorption sites, and the total amount of heavy metals in the soil (Brümmer *et al*. 1986; Hornburg and Brümmer 1993; Gray *et al*. 1999).

Following the harvest of metal-enriched plants, the hazardous biomass should be stored or disposed appropriately. Some authors have proposed that the weight and volume of the contaminated biomass can be further reduced by ashing, compaction and composting (Kumar *et al*. 1995; Raskin *et al*. 1997; Garbisu and Alkorta 2001; Garbisu *et al*. 2002). Bridgewater *et al*. (1999) and Koppolu *et al*. (2003) have reported the utilization of pyrolysis to separate heavy metals from hyperaccumulators. High cost of installation and operation can be a limiting factor for the treatment of plant disposal. One of the key aspects to the acceptance of phytoextraction pertains to the measurement of its performance, ultimate utilization of by-products and its overall economic viability

(Ghosh and Singh 2005).

A lot of basic research for the phytoremediation has been carried out in an attempt to understand how plants take up large quantities of metals, together with the mechanisms of metal translocation from roots to shoots, storage and detoxification. For the achievement of phytoremediation, various factors such as metal availability in soil, uptake by plants, transport and metal concentration in shoots and plant-microbe interactions are greatly responsible. Then, the ability to accumulate heavy metals varies between species and between cultivars within a species. Moreover, there has been no report directly demonstrating the utilization of phytoremediation by-products as animal feed for the potential additional income.

Therefore, the present study was undertaken with the following objectives:

1) To investigate the phytoremediation abilities of three tropical pasture grasses from two types of lead contaminated soils having different soil pH (Chapter 2)
2) To identify the pasture grasses that could perform the phytostabilization ability by liming (Chapter 3)
3) To study the chelate-assisted lead phytoextraction of pasture grasses (Chapter 4)
4) To study the utilization of lead contaminated forage as animal feed (Chapter 5)

CHAPTER 2

Effects of lead contamination in soils on dry biomass, concentration and amounts of lead accumulated in three tropical pasture grasses

Abstract

A greenhouse pot-experiment was conducted to evaluate the phytoremediation abilities of three tropical pasture grasses (signalgrass, napiergrass and atratum) in response to two types of soils (Kunigami-maji and Shimajiri-maji) contaminated with three levels of lead (0, 150 and 300 mg kg^{-1}). The results demonstrated that the dry biomass, lead concentration and the accumulated amounts were different among the plant species and between the soil types. The amounts of dry matter in three tropical pasture grasses grown on Kunigami-maji soil were higher than that on Shimajiri-maji soil. On both soils, lead concentration of roots was higher than that of shoots, and it was suggested that transportation of lead from roots to shoots was restricted in these plants. The lead accumulated amounts per plant grown on Kunigami-maji soil were higher than that on Shimajiri-maji soil. And, on Kunigami-maji soil, accumulation of lead was

relatively high in both shoots and roots of signalgrass and atratum, while, in napiergrass, the high level of lead was found only in roots. Amounts of lead extracted from the shoots of signalgrass, napiergrass and atratum grown on Kunigami-maji soil contaminated with the highest lead level of 300 mg kg^{-1} were 1.64, 0.17 and 0.92 mg plant^{-1}, respectively. As Kunigami-maji had lower soil pH than Shimajiri-maji, it can be suggested that lower soil pH may enhance lead bioavailability and uptake by the tropical pasture grasses. In conclusion, signalgrass and atratum could be useful for phytoremediation of lead contaminated soil, especially on Kunigami-maji soil.

Introduction

Heavy metal contamination of soils can cause a variety of environmental problems, loss of vegetation and ground water contamination. Lead is one of the most frequently encountered heavy metals in polluted soil environment. Lead contamination was caused by mining and smelting activities, burning of leaded gasoline, disposal of municipal sewage and industrial wastes enriched in lead as well as using lead-based paints, explosives and linings (Kabata-Pendias and Pendias 1984; Seaward and Richardson 1990; Chaney and Ryan 1994). Anthropogenic-sourced lead accumulates primarily in the surface layer of soils, and its concentration decreases with depth (Cecchi *et al*. 2008). The decontamination of these soils by engineering methods is a high costing project (Baker *et al*. 1991; Salt *et al*. 1995). In recent years, using plants to treat remediation of heavy metal-contaminated soils has received increasing attention (Chaney 1983; Cunningham and Berti 1993; Baker *et al*. 1994; Raskin *et al*. 1994). Phytoremediation is a promising technology in cleanup of polluted sites due to the properties of less destructive, low of cost and environmentally friendly nature (Wang *et al*. 2012).

To establish a cost-effective remediation technique, plants selected must be able to tolerate to high level of heavy metals, and stabilize heavy metals in soils by root activities of plants. Lead is known as non-essential elements for plants. Nevertheless, there are numerous investigations showing that various plant species have the ability to absorb lead by roots and translocate lead from roots to shoots (Reeves and Brooks 1983; Qureshi *et al*. 1985; Baker and Walker 1989).

It has been reported that some plant species are known as hyperaccumulators which can accumulate extremely high content of heavy metals without showing the drastic impact on their growth and development (Reeves and Brooks 1983; Brooks and Malaisse 1985; Baker and Brooks 1989; Xiong 1997). Over 500 plant species in 101 families have been reported as hyperaccumulators for a variety of metals, which have particularly high occurrence in the family of Brassicaceae (Kramer 2010). Because of its low growth rate and small biomass, this hyperaccumulator is not practically suited to phytoremediation of lead from contaminated soils. The choice of suitable plant species is greatly important for success of plant-based technology. Successful phytoextraction of lead depends on the identification of suitable plant species, bioavailability of the contaminant in the environmental matrix, root uptake, internal translocation of the plant, and plant tolerance to lead. Kulakow *et al*. (2000) suggested that grasses tended to be excellent candidates, because their fibrous rooting systems can stabilize soil particles and provide a large contacting area faced to root-soil contact. In this study, signalgrass, napiergrass and atratum were selected for their advantages, such as fast growth rate, high biomass, high resistance to adverse conditions, large root surface area, ease of cultivation and repeated cropping, which make them easy to be applied compared with many hyperaccumulators to be discovered. The present study aimed to investigate the lead uptake and translocation in three tropical pasture grasses and to identify species that have the ability to accumulate or stabilize the lead in the soil particles.

Materials and methods

The pot-culture experiment was conducted in the greenhouse at the Subtropical Field Science Center of University of the Ryukyus, Okinawa, Japan during the period from 11 May 2013 to 25 June 2013.

2.1 Preparation of experimental soils and chemical analysis

Two types of soils, red yellow soil (Kunigami-maji; KM) and dark red soil (Shimajiri-maji; SM) were used in this study. Five hundred grams of air-dried soils, sieved to 4 mm, were added into each Neubauer's pot (with an area of the base of 100 cm^2). As the soil chemical properties, soil pH, total lead contents and water soluble lead content of the experimental soils were determined in the study. The soil pH was determined in soil to distilled water ratio of 1:2.5 using a digital pH meter (Navi F51, Horiba Ltd., Kyoto, Japan). The total lead contents in soils were analyzed by an inductively coupled plasma atomic emission spectroscopy (ICP-AES) (ICPE-9000, Shimadzu Co.Ltd., Kyoto, Japan), following digestion with concentrated nitric acid. The water soluble lead content in soils was measured after extraction of 4 g of dry soil with 40 ml of deionized water in an orbital shaker for 6 hours, allowed for 10 minutes to settle. The water extracts were filtered through 0.45µm sieve (Advantec, Tokyo, Japan) into 15 ml polyethylene tube, and were analyzed by using ICP-AES.

2.2 Pot culture

The experimental design was a three-factor completely randomized design with three replicates. The factors were the soil types (KM and SM soils), the grass species (signalgrass, napiergrass and atratum) and the soil lead treatments. As the soil lead treatments, three treatments, which included the control (without adding lead) and two levels of lead treatment (150 and 300 mg kg^{-1}), representing as Pb 150 and Pb 300, respectively, were applied. Analytical chemical grade of $Pb(NO_3)_2$ as lead was supplemented to the soils. In signalgrass and atratum, 25 seeds per pot were sown evenly, while in napiergrass, a stem with two nodes was planted into each pot. All pots were watered daily to keep the soil moisture at 60–70% of field capacity. After germination, young seedlings were fertilized at the rate of 10 g N m^{-2}, 5.6 g P_2O_5 m^{-2} and 7.8 g K_2O m^{-2}. The air temperature in the greenhouse was regulated in the range of 25 to 35°C without humidity control.

2.3 Plant harvest and analysis

Forty five days after planting, plant samples were gently removed from the pots, and then separated into roots and shoots. The roots were washed firstly with tap water, followed by washing with deionized water, blotted dry on filter paper, and then dried at 70°C for 2 days to determine plant dry matter.

The dried plant samples of 0.5 g, ground to pass through the 0.5 mm sieve, were digested in concentrated nitric acid using a microwave laboratory system (Start D, Milestone General K.K. Co.Ltd., Kawasaki, Japan). All laboratory equipment (plastic and glass wares) were washed with distilled water, soaked in 1 mole of nitric acid (60%) overnight, rinsed with deionized water and air-dried before use. The digested solution was filled up to 100 ml final volume with deionized water, and filtered with 0.45 µm sieve (Advantec, Tokyo, Japan). Lead concentrations were analyzed using an ICP-AES, and lead accumulated amounts (mg plant^{-1}) were calculated by multiplying lead concentration in roots and shoots and plant dry matter to evaluate plant phytoextraction efficiency.

2.4 Statistical analysis

A three-way analysis of variance (ANOVA) was used for statistical analysis (SPSS version 16.0; U.S.A). Least significant difference (LSD) test was performed to define significant difference between specific mean pairs at a probability level of 0.05.

Results

The soil characteristics of the experimental soils were shown in Table 2.1. SM soil had a higher value of soil pH than KM soil. Total lead content in SM soil (227.80

mg kg^{-1}) was much higher than that in KM soil (25.72 mg kg^{-1}). However, water soluble lead contents were not much different between the two soils. Results of the three-way ANOVA were shown in Table 2.2. Root lead concentration was affected by the soil types and the soil lead treatments, with significant soil×grass and soil×lead treatment interactions. On the other hand, shoot lead concentration was affected by all sources of soils, grasses and lead concentrations, with significant two- and three-way interactions. The amounts of lead accumulated in roots and shoots were affected by all sources, with significant two- and three-way interactions (except for the three-way interaction in root lead accumulated amount).

Dry matter of the three grass species in response to increasing lead levels on two types of contaminated soils were shown for KM and SM soils in Figures 2.1 and 2.2, respectively. The differences in dry matter were not statistically significant among the soil lead treatments in any grass species grown on KM soil (Figure 2.1). On the other hand, on SM soil, the effect of lead treatment on dry matter was significant in signalgrass, but not in napiergrass and atratum (Figure 2.2).

Lead concentrations in roots and shoots of the three tropical grasses from different levels of lead contaminated KM soil were shown in Table 2.3. In the treatments of Pb 150 and Pb 300, all grasses examined had significantly higher lead concentrations in roots and shoots compared with the control. In the treatment of Pb 300, signalgrass and atratum attained an average lead concentration of 225.01 and 246.65 mg kg^{-1} in the shoots and 621.11 and 642.31 mg kg^{-1} in the roots, respectively, which were much higher than napiergrass (98.44 mg kg^{-1} in the shoots and 527.63 mg kg^{-1} in the roots). In SM soil, the lead treatment increased lead concentration in the roots of napiergrass, but not in the shoots (Table 2.4). Moreover, in signalgrass and atratum, neither root lead concentration nor shoot lead concentration was affected by the lead treatment.

The lead accumulated amounts (mg $plant^{-1}$) of the three tropical grasses with increasing soil lead levels from the two different soils were shown in Table 2.5. On KM soil, all species examined increased the lead accumulated amounts of roots with an increase in the soil lead treatment. Moreover, in signalgrass and atratum, the lead accumulated amounts of the shoots in Pb 150 and Pb 300 treatments were significantly higher than that in the control on KM soil. On the other hand, on SM soil, the effect of the soil lead treatment on the lead accumulation of the grass species was significant only in the shoots of signalgrass and in the roots of napiergrass. Comparing the results obtained on both soil types, the lead accumulated amounts per plant on KM soil were much higher than that on SM soil in all of the grass species examined.

Table 2.1 Selected chemical properties of the two experimental soils

Parameter	Kunigami-maji	Shimajiri-maji
pH	4.63	7.28
Total lead (mg kg^{-1} DW)	25.72	227.80
Water soluble lead (mg kg^{-1} DW)	4.23	4.87

Table 2.2 Three-way ANOVA summary for lead concentrations and accumulated amounts in roots and shoots of signalgrass, napiergrass and atratum from lead contaminated KM and SM soils

Name of source		Lead concentration ($mg\ kg^{-1}$)		Lead accumulated amounts ($mg\ plant^{-1}$)	
		Root	Shoot	Root	Shoot
Soil	KM	328.19	127.28	0.516	0.623
	SM	129.59	45.53	0.058	0.040
Grass	Signalgrass	211.78	87.15	0.343	0.516
	Napiergrass	211.31	67.42	0.172	0.090
	Atratum	263.58	104.65	0.347	0.388
Pb treatment†	Control	59.45	39.31	0.073	0.078
	Pb 150	227.04	103.27	0.310	0.437
	Pb 300	400.18	116.65	0.479	0.479
P value	Soil	***	***	***	***
	Grass	NS	**	***	***
	Pb treatment	***	***	***	***
	Soil×Grass	*	***	**	***
	Grass×Pb treatment	NS	*	*	***
	Soil×Pb treatment	***	***	***	***
	Soil×Grass×Pb treatment	NS	**	NS	***

NS Nonsignificant, *** Significant at P<0.001, ** Significant at P<0.01, * Significant at P<0.05

†Control: No lead added.

Pb 150, Pb 300: Lead contamination at 150 and 300 $mg\ kg^{-1}$ soil, respectively.

Table 2.3 Lead concentration (mg kg^{-1}) in roots and shoots of three tropical grasses under lead contaminated soil on Kunigami-maji

Species	Pb treatment†	Root	Shoot
Signalgrass	Control	72.97 c	23.78 b
	Pb 150	264.07 b	154.78 a
	Pb 300	621.11 a	225.01 a
Napiergrass	Control	55.63 c	20.13 b
	Pb 150	239.99 b	82.95 a
	Pb 300	527.63 a	98.44 a
Atratum	Control	49.33 b	35.23 b
	Pb 150	480.66 a	258.57 a
	Pb 300	642.31 a	246.65 a

a-c Mean values with different letters in the same column are significantly different between treatments for each species ($P<0.05$) by LSD test.

†Control: No lead added.

Pb 150, Pb 300: Lead contamination at 150 and 300 mg kg^{-1} soil, respectively.

Table 2.4 Lead concentration (mg kg^{-1}) in roots and shoots of three tropical grasses under lead contaminated soil on Shimajiri-maji

Species	Pb treatment†	Root	Shoot
Signalgrass	Control	61.95 a	48.96 a
	Pb 150	90.84 a	31.85 a
	Pb 300	159.74 a	38.50 a
Napiergrass	Control	92.53 c	41.85 a
	Pb 150	134.05 b	20.59 a
	Pb 300	225.46 a	25.03 a
Atratum	Control	67.16 a	65.90 a
	Pb 150	152.61 a	70.86 a
	Pb 300	224.86 a	66.25 a

a-c Mean values with different letters in the same column are significantly different between treatments for each species ($P<0.05$) by LSD test.

†Control: No lead added.

Pb 150, Pb 300: Lead contamination at 150 and 300 mg kg^{-1} soil, respectively.

Table 2.5 Lead accumulated amounts (mg plant^{-1}) of three tropical grasses from two different soils contaminated with different lead levels

Soil	Pb treatment†	Signalgrass		Napiergrass		Atratum	
		Root	Shoot	Root	Shoot	Root	Shoot
Kunigami-maji	Control	0.24 b	0.18 c	0.06 b	0.05 a	0.08 b	0.15 b
	Pb 150	0.56 b	1.12 b	0.30ab	0.19 a	0.85 a	1.18 a
	Pb 300	1.11 a	1.64 a	0.54 a	0.17 a	0.92 a	0.92 a
Shimajiri-maji	Control	0.02 a	0.03 b	0.02 b	0.02 a	0.02 a	0.04 a
	Pb 150	0.05 a	0.05ab	0.07 b	0.03 a	0.04 a	0.04 a
	Pb 300	0.08 a	0.07 a	0.14 a	0.03 a	0.08 a	0.04 a

a-c Mean values with different letters in the same column are significantly different between treatments for each species ($P<0.05$) by LSD test.

†Control: No lead added.

Pb 150, Pb 300: Lead contamination at 150 and 300 mg kg^{-1} soil, respectively.

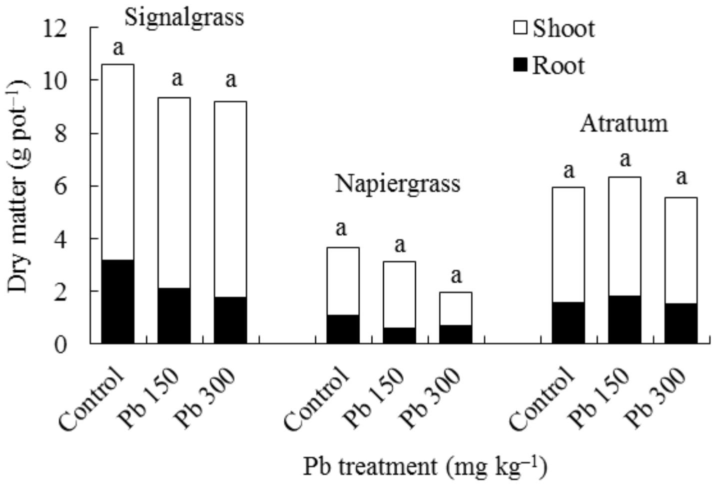

Figure 2.1 Root and shoot dry matter (g pot^{-1}) of signalgrass, napiergrass and atratum in response to different levels of lead (Pb) contaminated soil on Kunigami-maji.

Mean values with different letters in the same species are significantly different between Pb treatments ($P<0.05$) by LSD test.

Pb treatment : Control, Pb 150 and Pb 300 show 0 mg kg^{-1}, 150 mg kg^{-1} and 300 mg kg^{-1}, respectively.

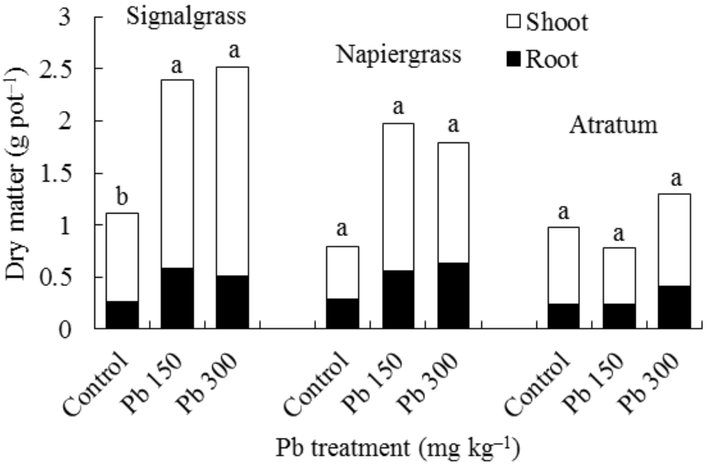

Figure 2.2 Root and shoot dry matter (g pot^{-1}) of signalgrass, napiergrass and atratum in response to different levels of lead (Pb) contaminated soil on Shimajiri-maji.

Mean values with different letters in the same species are significantly different between Pb treatments ($P<0.05$) by LSD test.

Pb treatment: Same as in Figure 1.

Discussion

Biomass would be a good parameter to assess heavy metals toxicity in soil (Ebrahimi 2012). It is generally accepted that trace amounts of heavy metals stimulate plant growth, while high concentrations of metals can cause damage on plant growth. Although most plant species demonstrated an overall dose dependent response to the contaminated soil, an increase in root and shoot dry biomass was evident at some lead concentrations. The increase in dry biomass caused by the lead treatment was observed in signalgrass and napiergrass grown on SM soil (Figure 2.2). The response pattern of the plants with application of $Pb(NO_3)_2$, according to the dry biomass, seemed to be a combination of some stimulating and inhibitory effects (Dou and Hu 1987; Xiong 1998). It is reported that lead has some stimulating factor, or some other substances rather than lead were responsible for stimulating plant growth (Kabata-Pendias and Pendias 1984). It is suggested that stimulating effect on the plant growth of signalgrass and napiergrass by adding nitrate with the addition of lead had overcome the inhibitory effect up to the soil lead treatment of 300 mg kg^{-1} on SM soil. Similar stimulating phenomenon of lead treatments to plant growth was reported by Xiong (1998). Although no matter which mechanism is involved in the stimulation of plant growth under the condition of lead supplemented to the soils, lead contaminated KM soil exhibited higher total dry matter than that on SM soil. It could be considered that the tropical pasture grasses in this study seemed likely to be more adaptive to acidic KM soil compared with SM soil.

The rate of lead uptake by plant is substantially affected by plant species grown on different soils (Bosque *et al*. 1990; Tlustoš *et al*. 2001). The data in this study revealed that signalgrass and atratum had higher lead concentrations in the roots and shoots than napiergrass in KM soil (Table 2.3). On the other hand, in SM soil, since lead concentrations of the roots were much higher than those of the shoots, it was suggested that only a few was transported into the shoots (Table 2.4). The factors determining heavy metals distribution in different parts of plants may depend on the metal translocation process in plants. Regarding metal concentration in shoots, roots had more important role for the absorption of metals. There are similar analytical data for other species which suggest some restrictions in the transport of metals from roots to shoots: the restrictions include immobilization by negatively charged pectins within the cell wall (Islam *et al*. 2007; Kopittke *et al*. 2007; Arias *et al*. 2010), precipitation of insoluble lead salts in intercellular spaces (Islam *et al*. 2007; Kopittke *et al*. 2007; Malecka *et al*. 2008; Meyers *et al*. 2008), accumulation in plasma membranes (Seregin *et al*. 2004; Islam *et al*. 2007; Jiang and Liu 2010), or sequestration in the vacuoles of rhizodermal and cortical cells (Seregin *et al*. 2004; Kopittke *et al*. 2007). In addition, in this study, relatively small increases were found in the lead concentrations in the shoots of napiergrass grown on soils even with large additions of soluble lead salts. Similar results were reported by Baumhardt and Welch (1972), who mentioned that large amounts of lead added into soil did not show an increase in lead concentration in corn plants. These observations are all consistent with the concept that roots provide a barrier which restricts the movement of lead through the plant (Lagerwerff 1971; Baumhardt and Welch 1972; Jones and Clement 1972).

The lead hyperaccumulator plants are defined as those, in which lead concentration in the shoot dry matter exceed threshold of 1000 mg kg^{-1} (Baker 1981; Baker and Brooks 1989). Tamura *et al*. (2005) have discovered that common buckwheat (*Fagopyrum esculentum* Moench), the first known lead hyperaccumulator species, can naturally accumulate up to 4200 mg kg^{-1} of lead in the shoot. Xiong (1998) reported that *Brassica pekinensis* plants were capable of translocating higher concentrations of lead to aerial plant parts (1445 mg kg^{-1} at 250 mg kg^{-1} soil lead treatment) without incurring damage to their basic metabolic functions. Kumar *et al*. (1995) indicated that *Brassica juncea* accumulated large amounts of lead in shoots, and the lead concentrations in roots and shoots varied greatly among different *B. juncea* cultivars, as this species may have a large genetic variability to accumulate lead. Although lead concentration of the pasture grass species in this experiment did not reach the optimal ability of lead hyperaccumulator plants, signalgrass and atratum indicated the relatively high lead uptake in their roots and shoots on KM soil (Table 2.3). Furthermore, the characteristics such as easy cultivation and repeated cropping of grasses can enable the phytoremediation of lead contaminated soils by repeated harvesting of the aboveground plant parts.

The knowledge about the abilities of different plant species or tissues to absorb and transport metals under different conditions will provide insight into choosing appropriate plants for phytoremediation in the polluted regions. The plant uptake of heavy metals from soil is also correlated with soil metal concentration in the soluble fraction. Lead is usually very insoluble (not available for plant uptake) in the normal range of soil pH. The solubility of lead increases at soil pH below 4.5 (Hornburg and Brümmer 1993). The difference in the two tested soils also suggests that it is important to identify soil characteristics considering soil pH as a strategy to enhance phytoextraction.

The present study demonstrated that lead concentrations in shoots and roots of napiergrass were much less than those of the other two species in KM soil. In SM soil, however, three species indicated lower lead concentrations in their roots and shoots compared with KM soil (Tables 2.3 and 2.4). The low bioavailability of lead could also be due to the relatively high pH in the contaminated SM soil. The lead concentrations of napiergrass shoots did not show much difference in both soils. Therefore, in this study, it can be considered that napiergrass had no tolerance at both lead soil contamination and also a minimum pH of 4.63.

It has been recognized that, the lead concentrations in shoots of signalgrass were lower than those of atratum in KM soil (Table 2.3). High dry matter was compensated by the moderate lead concentrations of signalgrass and the lead accumulated amount in shoots of signalgrass was higher than that of atratum in KM soil (Table 2.5). Similarly, high dry matter and higher lead concentration in roots of napiergrass in SM soil showed the highest lead accumulated amounts among the three grass species (Tables 2.4 and 2.5). However, the amounts of lead accumulated in the harvestable parts of plants were the most important for phytoextraction of lead contaminated soils (Khan *et al*. 2000).

The data of the present experiment indicated that lead uptake by the grass species examined was more efficient in KM acid soil than in SM alkaline soil. It is suggested that the lower soil pH can enhance lead bioavailability and uptake of the tropical pasture grasses. Aluminum (Al) toxicity, which limits plant growth, occurs at low soil pH and then a plant species tolerant to Al is highly desirable for phytoextraction. Signalgrass has been reported to have an outstanding level of Al resistance compared with Al-resistant genotypes of graminaceous crops such as wheat, triticale and maize (Wenzl *et al*. 2001). Signalgrass was able to grow in KM soil with high dry matter and high lead concentration in the shoots. High biomass and high lead concentration of atratum in both soils seemed to be quite tolerant to soil pH, but the values in SM soil were lower than that in KM soil. Lead concentrations in the shoots of napiergrass were quite low, while those in roots were extraordinary high. Consequently, napiergrass may be regarded as a candidate species for phytostabilization of lead pollution, which not only beautifies the environment, but also reduces the risk of food chain pollution (Mclntyre 2003). Clemens *et al.* (2002) reported that an ideal plant for heavy metal phytoextraction should grow fast, have high biomass and deep root system, be easy to harvest, and be able to tolerate and accumulate a range of heavy metals in the harvestable components.

Conclusion

In this study, signalgrass and atratum showed the ability to uptake lead without any marked negative effect on dry biomass, and the capability to accumulate high concentration of lead in their shoots could be useful for phytoextraction of lead contaminated soils, especially on Kunigami-maji soil.

CHAPTER 3

Effects of liming on dry biomass, lead concentration and accumulated amounts in roots and shoots of three tropical pasture grasses from lead contaminated acidic soils

Abstract

Liming the contaminated soil is the most widely used remediation treatment to reduce the bioavailability of heavy metals. The objective of this study was to evaluate the effect of liming on the change of dry matter and lead uptake by three tropical pasture grasses from lead contaminated acidic soil. Lime at five rates of 0, 1, 2, 3 and 4 g kg^{-1} soil was amended to the Neubauer's pots filled with 500 g Kunigami-maji soils and then the limed soil was contaminated with 150 mg kg^{-1} lead after it was maintained for one week. Addition of lime increased soil pH significantly from 4.43 to 5.40. The root and shoot dry matter of all the three tropical pasture grasses increased with the increasing doses of lime. An elevation of soil pH induced by liming resulted in a significant reduction of lead concentrations in both roots and shoots of all experimental grasses.

The effectiveness of liming on lead concentration and accumulation varied with the pH values of limed soil and grass species. The results of this study implied that napiergrass was the most effective tropical pasture grass in reducing lead concentration and accumulation of roots and shoots as a consequence of liming, and could be used for lead stabilization in moderately lead contaminated acidic soil. The shoot lead concentration of napiergrass in limed soils was within the critical level of lead tolerable to feeding domestic animals, and may act as low level lead toxicity in fodder for grazing livestock. However, lime application or soil pH had a little influence on the lead accumulated amounts in roots and shoots of atratum and signalgrass. And, the high amounts of lead accumulated in shoots of atratum and signalgrass were found to be useful for lead phytoextraction.

Introduction

During the past few decades, industrial activities and improper use of chemical fertilizer and pesticides, industrial effluents, sewage sludge and wastewater irrigation have resulted in an increasing number of heavy metal-contaminated sites around the world (Kuo *et al*. 2006; Ramadan and Al-Ashkar 2007). Lead is considered as a potential human health hazardous heavy metal, which may pose a great threat to plants, animals and humans through the food chain (Connell and Miller 1984; Han *et al*. 2006). Therefore, it is necessary to clean the contaminated areas to remediate polluted soils and to reduce toxic metals in the food chain. There are some conventional remediation technologies to clean up the polluted soils, however, these methods are expensive, time-consuming and environmentally devastating (Liu *et al*. 2010). In recent years, the emerging phytoremediation technologies with less-destructive, low-cost and environmentally friendly nature, have received increasing attention (Garbisu and Alkorta 2001). Phytoremediation of heavy metal-contaminated soils basically includes phytostabilization and phytoextraction with the former using plants to retain the metals in the roots or within the rhizosphere by restricting their translocation to above-ground parts for the less bioavailability of pollutants into the environment, and the latter using plants to extract contaminants from soil into plants (Wong 2003; Alkorta *et al*. 2004).

Some soils are highly contaminated and it would take a considerably longer period of time for phytoextraction of heavy metals from such soils. If such soils are not remediated, these could be a major source of heavy metal dispersion into the environment. The risk posed by such soils can be reduced by using plants to immobilize the metals in the soil (Marques *et al*. 2009). A common method for immobilization of metals in soils is to apply lime, phosphates or organic matter residues (Bolan and Duraisamy 2003). There is a common understanding reported in the literatures that soil pH is one of the most important factors determining the concentration of metals in soil solution, their mobility and availability to plants (Alkorta *et al*. 2004; Wang *et al*. 2006; Domańska and Filipek 2011). Lime application is a part of normal cultural practices in acidic soils, to increase soil pH as well as to decrease heavy metals concentration in above-ground parts of plants (Han *et al*. 2007). However, some case studies indicated that addition of lime or changes in soil pH was generally ineffective in the concentrations of lead in plants (Sims and Kline 1991; Han and Lee 1996; Hooda and Alloway 1996).

Therefore, the selection of appropriate plant species in combination with lime application is imperative to estimate the uptake of lead by plants. When some grass species are introduced in pasture area, it is necessary to identify a suitable species which can function as phytostabilization or phytoextraction under lead contaminated soils. Because some former species could retain lead in the roots or within the rhizosphere, tropical pasture grass species could be cultivated by liming. In this study, signalgrass, napiergrass and atratum were selected mainly for their deep and fibrous rooting system. The present work aimed to study the changes of soil pH, dry matter, lead uptake and translocation in three tropical pasture grasses as a consequence of lime application to lead contaminated acidic soil.

Materials and methods

A pot culture experiment was conducted in the greenhouse at the Subtropical Field Science Center of the University of the Ryukyus, Okinawa, Japan during the period from 12 August 2013 to 26 September 2013.

3.1 Preparation for soils and experimental design

Red yellow soil (Kunigami-maji; KM) was used in this study. Five hundred grams of air-dried soils, sieved to 4 mm, were added into each Neubauer's pot (with a base area of 100 cm^2). The effect of liming was tested using calcium carbonate ($CaCO_3$) for increasing soil pH. Five treatments with three replications were allocated, one control (without the addition of lime) and four levels of lime (1, 2, 3 and 4 g kg^{-1}) given to the pots. After the addition of lime, the soils were maintained to adjust pH for one week. The soil pH, total lead and water soluble lead contents of the experimental soil were determined in the study by the use of methods described in Chapter 2.

The pots were arranged randomly with maintaining a constant irradiated condition for each pot. In each pot soil, lead (Pb) as analytical chemical grade of $Pb(NO_3)_2$ was added at the rate of 150 mg Pb kg^{-1}, then mixed thoroughly with the soil. Lead was added once prior to the experiment. Planting preparation, fertilizer application and maintaining soil moisture were performed as described in Chapter 2.

3.2 Plant harvest and analysis

The plant sample preparation and data analysis were carried out as described in Chapter 2.

3.3 Statistical analysis

All data were tested for normal distribution with Shapiro-Wilk test. A two-way analysis of variance (ANOVA) – lime application (5 levels) and tropical pasture grasses (3 species) – was used to analyze dry matter, lead concentration and accumulation in the roots and shoots. ANOVA was followed by least significant difference (LSD) test for multiple comparisons. All statistical analyses were conducted with SPSS version 16.0 (SPSS Chicago, IL, U.S.A).

Results

The experimental soil characteristics were as follows: soil pH of 4.43, total lead content of 28.55 mg kg^{-1} and water soluble lead content of 4.36 mg kg^{-1}. Soil pH values after the harvest of tropical grass species in soils treated with different amounts of $CaCO_3$ were presented in Table 3.1. The soil pH was significantly increased from 4.43 to 5.40 by the addition of lime ($P<0.05$).

Two-way ANOVA results revealed that the root and shoot dry matter were significantly differed with both grasses and lime levels applied to the acidic soil, showing no significant interaction between grass species and lime levels (Table 3.2). Signalgrass showed the highest root and shoot dry matter (2.86 and 7.93 g pot^{-1}), followed by atratum (1.52 and 4.37 g pot^{-1} in root and shoot) and napiergrass (1.40 and 4.45 g pot^{-1} in root and shoot) (Table 3.3). The shoot and root dry matter increased significantly with the increase in lime levels (Table 3.4).

According to the results of two-way ANOVA, the lead concentrations in roots were significantly affected only by lime levels, while those in shoots were significantly affected by both grasses and lime levels, with no significant interaction between grasses and lime levels (Table 3.2). Increasing lime application significantly reduced lead concentrations in roots and shoots of grass species (Table 3.4). Atratum showed the higher shoot lead concentration than napiergrass and signalgrass (Table 3.3).

The lead accumulation in the plants was significantly affected by both the grass species and the lime levels supplied in the KM acidic soil as well as by the interaction between the two factors (Table 3.2). The amounts of lead accumulated in roots and shoots of grass species in response to the changes of soil pH were shown in Figures 3.1 and 3.2, respectively. The amounts of root and shoot lead accumulated in signalgrass (mg plant^{-1}) were the highest in lower soil pH and decreased sharply with the increasing soil pH. However, the initial high lead accumulations in roots of napiergrass were found with the soil pH 4.57–4.78, and those in shoots were between 4.43 and 4.57, followed by a slow reduction with increasing soil pH. The higher amounts of accumulated lead in the root and shoot of atratum were found with the soil pH 4.57–5.03, followed by a decrease in lead accumulation with increasing soil pH.

Table 3.1 Soil pH of three tropical pasture grasses after 45 days of lead contaminated soil treated with different amounts of $CaCO_3$

Treatment (g kg^{-1} $CaCO_3$ soil)	Soil pH (in H_2O)
0	4.43 d
1	4.57 d
2	4.78 c
3	5.03 b
4	5.40 a

a-d Mean values with different letters are significantly different from one another ($P<0.05$) by LSD test.

Table 3.2 Results of two-way ANOVA

Source of variation	df	Computed F											
		Dry matter				Lead concentration				Lead accumulation			
		Root		Shoot		Root		Shoot		Root		Shoot	
Grasses (A)	2	18.38	***	27.50	***	1.36	NS	25.53	***	13.83	***	86.79	***
Lime levels (B)	4	3.34	*	6.52	**	12.39	***	9.52	***	7.07	***	21.94	***
A×B	8	0.53	NS	1.21	NS	0.38	NS	1.89	NS	8.39	***	19.93	***

NS Nonsignificant

*** Significant at P<0.001

** Significant at P<0.01

* Significant at P<0.05

Table 3.3 Comparison of dry matter, lead concentration and accumulated amounts among grass species

Species	Dry matter ($g\ pot^{-1}$)		Lead concentration ($mg\ kg^{-1}$)		Lead accumulated amounts ($mg\ plant^{-1}$)	
	Root	Shoot	Root	Shoot	Root	Shoot
Signalgrass	2.86 a	7.93 a	177.02 a	71.47 b	0.49 a	0.53 a
Napiergrass	1.40 b	4.45 b	195.60 a	23.11 c	0.24 b	0.08 b
Atratum	1.52 b	4.37 b	237.05 a	167.30 a	0.30 b	0.58 a

a-c Mean values with different letters in the same column are significantly different at $P<0.05$ level by LSD test.

Table 3.4 Comparison of dry matter, lead concentration and accumulated amounts from different lime levels

Lime levels (g $CaCO_3$ kg^{-1} soil)	Dry matter (g pot^{-1})		Lead concentration (mg kg^{-1})		Lead accumulated amounts (mg plant^{-1})	
	Root	Shoot	Root	Shoot	Root	Shoot
0	1.22 b	3.41 b	406.78 a	178.86 a	0.48 a	0.41bc
1	2.29 a	6.22 a	202.52 b	106.68 b	0.45ab	0.66 a
2	2.09 a	5.93 a	166.82bc	66.80bc	0.32bc	0.42 b
3	2.27 a	6.73 a	138.18bc	49.50 c	0.29cd	0.31 c
4	1.77ab	5.64 a	101.80 c	34.62 c	0.17 d	0.18 d

a-d Mean values with different letters in the same column are significantly different at $P<0.05$ level by LSD test.

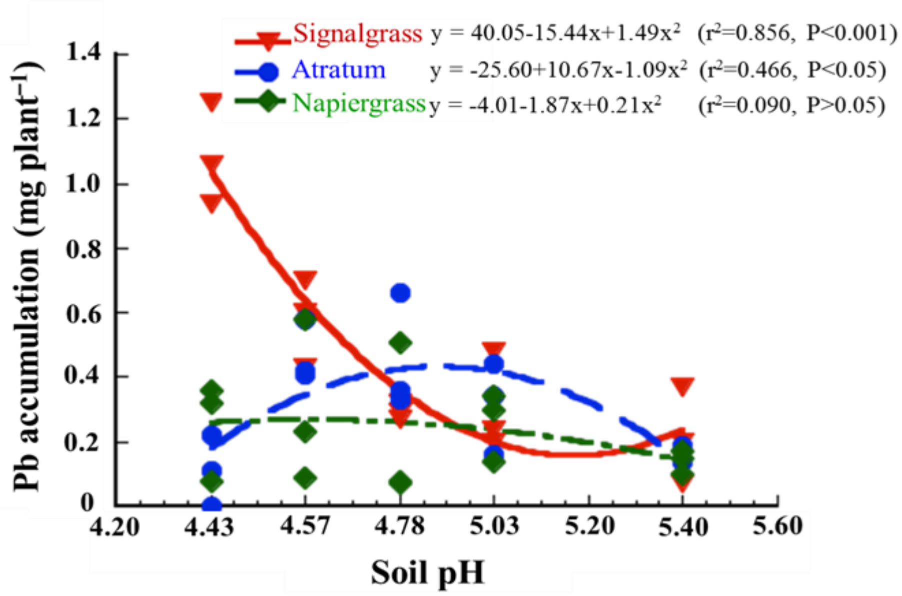

Figure 3.1 Lead accumulation (mg plant^{-1}) in roots of three tropical pasture grasses in response to the changes of soil pH.

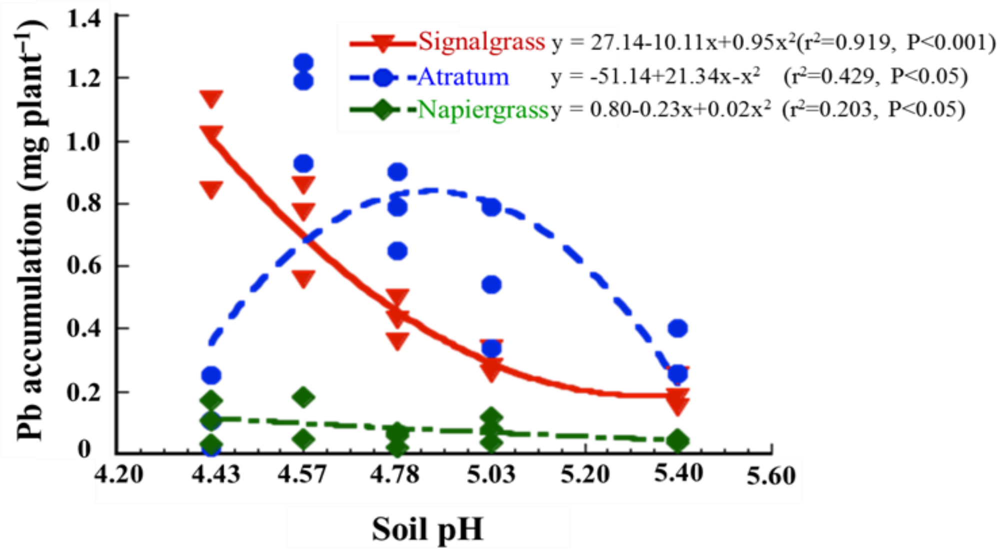

Figure 3.2 Lead accumulation (mg plant^{-1}) in shoots of three tropical pasture grasses in response to the changes of soil pH.

Discussion

Soil pH is the most important property that controls mobility and phytoavailability metals in soils directly or indirectly. Application of calcium carbonate is a traditional practice in acidic soil to increase soil pH. This practice not only increases soil pH but also reduces plant available heavy metals by a cation exchange process (Guo *et al*. 2006). In this study, the application of lime increased soil pH in a range of 0.2 to 1.0 unit, correspondingly to pH 4.57 to 5.40 (Table 3.1). The rise in soil pH induces metal immobilization because it favors metal precipitation, decreases metal solubility and promotes metal adsorption by increasing the net negative charge of variably charged soil constituents (Lindsay 1979; McBride *et al*. 1997; Bradl 2004). Addition of lime or increasing soil pH caused higher reduction of lead concentration in roots and shoots of the three tropical pasture grasses (Table 3.4). These results were consistent with the findings of other studies (Cox and Rains 1972; Han *et al*. 2007). Calcium released from lime followed by plant absorption could inhibit the translocation of metals including lead from roots to shoots. Although the lead concentrations in roots were not much different among the examined pasture grasses, those in shoots varied greatly among the grass species. Napiergrass and signalgrass retained high amounts of lead in roots and restricted lead transport to shoots (Table 3.3). Zimdahl and Foster (1976) found that liming reduced lead translocation from roots to shoots of corn. The shoot lead concentration of napiergrass in limed soils was less than 30 mg kg^{-1}, which notified the lead tolerable level for domestic animals in air dried forage by NRC (1980). Hooda *et al* (1997) reported that liming soil reduced heavy metal concentrations in plants, however, plant availability of heavy metals differed widely among the plant species. The present study confirmed that the effectiveness of liming varied with the pH values of limed soil and plant species.

Although the increased application of lime was effective in reducing lead concentrations in the shoots of grass species used, the higher lead accumulated amounts in the shoots were shown in some lime levels (1 and 2 g $CaCO_3$ kg^{-1} of soil, which corresponds to soil pH 4.57 and 4.78). Atratum had the higher amounts of accumulated lead with the soil pH 4.57–5.03 (Figures 3.1 and 3.2). Since the amounts of accumulated lead in the plants were calculated by multiplying the lead concentrations and the plant dry weight, high dry matter in some limed levels compensated for the moderate lead concentrations in the grass species used for all experiments. Though signalgrass showed a continuous lead reduction with an increase in soil pH, its shoot lead accumulated amount was 0.53 mg $plant^{-1}$, which was higher than that of napiergrass. Atratum had the highest lead accumulated amounts (0.58 mg plant^{-1}). It could be suggested that adaptive soil pH level for each grass species might vary for optimal dry matter production and lead accumulated amounts, even though lead concentrations in all grasses were reduced with increased soil pH or the addition of lime. Little effect of lime application on lead accumulated amounts in signalgrass and atratum was found in this study.

Conclusion

Napiergrass maintained high lead concentrations in the roots and low lead concentrations in the shoots, while showing a slow reduction of lead accumulated amounts with increasing soil pH. Consequently, this species may be considered as a candidate one for phytostabilization of lead contamination, which not only purifies the contaminated soil, but also reduces lead toxicity in fodder for livestock grazing or feeding. Signalgrass and atratum had the high lead accumulated amounts in shoots, which could be useful for lead phytoextraction.

CCHAPTER 4

Effects of EDTA and citric acid on dry biomass, lead concentration and accumulated amounts in *Brachiaria decumbens* and *Paspalum atratum* from lead contaminated acidic soils

Abstract

A pot experiment was conducted to evaluate the effects of EDTA and citric acid on the uptake of lead by using high biomass plants (signalgrass and atratum). Application levels (0, 1.5, 2.5, 5 and 10 mmol kg^{-1} soil) of EDTA and citric acid were added to 150 mg kg^{-1} of lead contaminated soil one week before harvesting. The experimental period was 45 days. The results showed that signalgrass was able to grow in the presence of EDTA and citric acid showing no visible symptoms of phytotoxicity and it could have the ability of metal tolerance. EDTA (1.5, 2.5 and 5 mmol kg^{-1}) treated soil significantly increased the concentrations of lead in the shoots of signalgrass by 1.4, 1.5 and 1.3-fold, respectively, in comparison with the control and were clearly more effective in stimulating the translocation of lead from roots to shoots. In atratum,

the control plants were more efficient in the uptake and translocation of lead than when EDTA and citric acid were added. Two investigated grass species did not show the same results to the applied chelates. It is imperative to note that the plant species, chelator source and level will make a difference in uptake and translocation of lead. Both EDTA and citric acid were ineffective as an amendment to enhance the lead phytoextraction of atratum. Signalgrass showed comparative high dry matter while accumulating high concentrations of lead in their shoots and then could be suggested as a suitable candidate for chelate-induced phytoextraction of lead.

Introduction

Phytoextraction of heavy-metal contaminated soils is defined as the use of green plants to transport and concentrate metals from the soils into the above-ground shoots, which are harvested with conventional agricultural methods (Baker *et al*. 1994; Raskin *et al*. 1994; Kumar *et al*. 1995). Application of plants is much less expensive and less invasive for the environment (Cunningham and Berti 2000). Unfortunately, phytoremediation techniques are very time-consuming and their effects are visible only after several years. Plants for phytoextraction should have the following characteristics: (1) be tolerant to high levels of the metal, (2) accumulating reasonably high levels of the metal in their above-ground tissues, (3) rapid growth rates, (4) producing reasonably high biomass, and (5) having profuse root system (Clemens *et al*. 2002; Alkorta *et al*. 2004). Based on these facts, more recent research projects on phytoextraction have focused on high biomass plant species, such as maize (*Zea mays*), peas (*Pisum sativum*), oats (*Avena sativa*), barley (*Hordeum vulgare*) and Indian mustard (*Brassica juncea*), and on relevant plant husbandry and soil management practices to enhance the metal uptake of these species (Blaylock *et al*. 1997; Huang *et al*. 1997; Ebbs and Kochian 1998; Shen *et al*. 2002; Chen *et al*. 2004). Although several conditions must be met in order to achieve this technique, the lower metal bioavailability in the soil and poor metal translocation from roots to shoots are major limiting factors for phytoextraction of metals from polluted-soils (Huang *et al*. 1997; Epstein *et al*. 1999).

For example, lead (Pb), one of the most important environmental pollutants, has limited the solubility in soils and availability for plant uptake due to complexation with organic matter, sorption on oxides and clays, and precipitation as carbonates, hydroxides and phosphates (McBride 1994). Many plants retain lead in their roots with only minimal transport to the above-ground harvestable portions (Salt *et al*. 1995). Increased solubility can be achieved by adding chelates to the soil. Chelate-induced phytoextraction can be used for enhancing the uptake and translocation of metals in plants (Huang *et al*. 1997; Turgut *et al*. 2004).

Ethylenediamine tetraacetic acid (EDTA) is probably the most efficient chelate in increasing the concentration of various metals in above-ground plant tissues (Cunningham and Ow 1996; Blaylock *et al*. 1997; Huang *et al.* 1997; Vassil *et al*. 1998). EDTA increases not only the amount of soil lead taken up by plants but also metal transport through the xylem and lead translocation from roots to shoots and leaves (Huang *et al*. 1997; Epstein *et al*. 1999). Although EDTA is very effective in mobilizing metals in soils, EDTA and metal-EDTA complexes can be toxic to plants and soil microorganisms and they can also be persistent in the environment due to their low biodegradability (Lombi *et al.* 2001).

The use of natural compounds such as low molecular weight organic acids (LMWOA) which are easily biodegradable sounds better than synthetic chelate application to the public acceptance of phytoextraction technology. Application of LMWOAs like citric acid has been well documented for mobilizing heavy metals in soils and increasing their uptake by plants (Huang *et al*. 1998). But many authors have found lower effectiveness of LMOWAs such as citric acid in inducing metals accumulation in plants compared to synthetic chelates (Wu *et al*. 2004; Evangelou *et al*. 2006).

Therefore, it has been recognized that the selection of appropriate plant materials and chemical amendments is still very important even today for promoting phytoremediation efficiency. The present study focused on determining the effects of chelates (EDTA and citric acid) on the uptake of lead by signalgrass and atratum from lead contaminated acidic soils.

Materials and methods

A pot culture experiment was conducted in the greenhouse at the Subtropical Field Science Center of the University of the Ryukyus, Okinawa, Japan during the period from 16 May 2016 to 30 June 2016.

4.1 Preparation for soils and experimental design

Red yellow soil (Kunigami-maji; KM) was used in this study. The air-dried soils were sieved to 4 mm and added 500 g of soil into each Neubauer’s pot (with an area of the base of 100 cm^2). The subsets of pots for each species were treated with EDTA and citric acid in a single application to the surface of the soil at the rates of 0, 1.5, 2.5, 5 and 10 mmol chelate kg^{-1} soil one week before harvesting. The treatments were replicated three times. The pots were arranged randomly while maintaining a constant irradiated condition for each pot. The soil pH, total lead and water soluble lead contents of the experimental soil were determined in the study by the use of methods described in Chapter 2.

Twenty-five seeds each of signalgrass and atratum were sown separately to each pot. After germination, young seedlings were fertilized at the rate of 10 g N m^{-2}, 5.6 g P_2O_5 m^{-2} and 7.8 g K_2O m^{-2}, respectively. All pots were watered daily to keep the soil moisture at 60–70% of the field capacity throughout the experiment.

4.2 Plant harvest and analysis

The plant sample preparation and data analysis were carried out as described in Chapter 2. Lead accumulated amounts (mg $plant^{-1}$) of each experimental grass species were calculated by multiplying lead concentration in roots and shoots and respective plant dry matter weight. Additionally, translocation factor (TF) as the ratio of lead concentration in the shoot to that in the root can be used to evaluate the capacity of a plant to translocate lead from roots to shoots (Santos *et al*. 2006).

4.3 Statistical analysis

One-way analysis of variance (ANOVA) was used for statistical analysis (SPSS version 16.0; Chicago, U.S.A). Least significant difference (LSD) test was performed to define significant differences between specific mean pairs at a probability level of 0.05.

Results

The experimental lead contaminated soil characteristics were as follows: soil pH of 4.65, total lead content of 178.61 mg kg^{-1} and water soluble lead content of 7.10 mg kg^{-1}. Soil pH values after the harvest of tropical grass species in soils treated with different amounts of chelates were presented in Table 4.1. The soil pH was significantly decreased from 4.65 to 3.52 and from 4.65 to 4.50 by the addition of both EDTA and citric acid, respectively.

The dry matter of signalgrass and atratum grown on lead contaminated soils with chelate treatments was shown in Table 4.2. When no chelates were added to the soil, both grasses showed the highest shoot dry matter weight without visual symptoms of metal toxicity. EDTA concentrations gradually inhibited the shoot dry matter of the two plant species. In signalgrass, the root dry matter increased at some EDTA and citric acid concentrations. The citric acid application levels ranging from 2.5 to 10 mmol kg^{-1} soil significantly decreased root and shoot dry matter in atratum. And the addition of 10 mmol kg^{-1} soil citric acid to atratum caused chlorosis of leaves at the end of the experiment. The addition of EDTA appeared to be less toxic to both species of grasses compared with that of citric acid.

The lead concentration in roots and shoots of signalgrass and atratum with chelate treatments was shown in Table 4.3. The EDTA doses ranging from 1.5 to 5 mmol kg^{-1} soil significantly increased the lead concentration in the shoots of signalgrass. On the other hand, the addition of EDTA significantly decreased shoot lead concentrations in atratum. In signalgrass, increasing citric acid doses made an increase in lead uptake, however, the addition of 1.5 mmol kg^{-1} soil citric acid showed the highest shoot lead concentrations (156.57 mg kg^{-1}) among the treatments. In atratum, the lead concentration was lowered from 245.39 to 161.69 mg kg^{-1} in the shoot, and from 456.44 to 377.33 mg kg^{-1} in the root with citric acid amendments.

Although all EDTA application levels led to higher values of the TF in the signalgrass, the addition of 1.5 and 2.5 mmol kg^{-1} soil EDTA was clearly more effective in stimulating the translocation of metals from roots to shoots (Figure 4.1). In atratum, TFs were not much different in the presence of EDTA. Citric acid addition led to lower values of the TF in the signalgrass and atratum (except in 1.5 mmol kg^{-1} soil for signalgrass) (Figure 4.2).

Applying to signalgrass at the rates from 1.5 to 5 mmol kg^{-1} soil EDTA caused an enhanced shoot lead accumulation (Table 4.4). At a dose of 1.5 mmol kg^{-1} soil citric acid, shoot lead accumulation increased up to 0.86 mg $plant^{-1}$ in signalgrass. In atratum, the control (the absence of chelate treatments) showed the highest lead accumulated amounts in the shoots while, EDTA and citric acid amendments caused a significant reduction in shoot lead accumulation compared with the control.

Table 4.1 Soil pH after the harvest of pasture grasses on soils treated with different amounts of chelates

Treatment (mmol kg^{-1} soil)	Soil pH	
	EDTA	Citric acid
0	4.65 a	4.65 a
1.5	4.54 b	4.54 ab
2.5	4.50 b	4.54 ab
5	4.35 c	4.56 ab
10	3.52 d	4.50 b

a-d Mean values with different letters in the same chelate treatments indicate a significant difference $P<0.05$ according to LSD test.

Table 4.2 Dry matter (g pot^{-1}) in roots and shoots of signalgrass and atratum grown on lead contaminated soils with chelate treatments

Treatment	Signalgrass		Atratum	
(mmol kg^{-1} soil)	Root	Shoot	Root	Shoot
EDTA				
0	1.43 cd	6.77 a	1.42 a	3.39 a
1.5	2.49 a	5.94 a	1.22 ab	2.68 a
2.5	2.10 ab	5.37 a	1.36 ab	2.74 a
5	1.77 bc	6.35 a	1.23 ab	2.70 a
10	1.25 d	6.69 a	0.83 b	2.34 a
Citric acid				
0	1.43 b	6.77 a	1.42 a	3.39 a
1.5	1.13 b	5.57 b	1.51 a	3.18 ab
2.5	2.01 a	6.09 ab	0.95 b	2.08 c
5	1.15 b	5.62 b	0.93 b	2.53 bc
10	2.07 a	5.51 b	0.66 b	1.73 c

a-d Mean values with different letters in the same species and chelate treatments within a column indicate a significant difference $P<0.05$ according to LSD test.

Table 4.3 Lead concentration (mg kg^{-1}) in roots and shoots of signalgrass and atratum grown on lead contaminated soils with chelate treatments

Treatment (mmol kg^{-1} soil)	Signalgrass		Atratum	
	Root	Shoot	Root	Shoot
EDTA				
0	228.54 ab	87.79 b	456.44 a	245.39 a
1.5	195.43 c	122.48 a	298.98 ab	180.75 b
2.5	206.94 bc	131.58 a	386.51 ab	183.75 b
5	237.09 a	113.25 a	371.99 ab	102.24 c
10	192.58 c	84.34 b	251.99 b	134.89 c
Citric acid				
0	228.54 a	87.79 b	456.44 abc	245.39 ab
1.5	362.33 a	156.57 a	545.62 a	190.13 ab
2.5	292.38 a	96.00 b	377.33 c	180.45 ab
5	367.10 a	110.48 ab	397.55 bc	161.69 b
10	380.13 a	90.40 b	520.83 ab	251.96 a

a-c Mean values with different letters in the same species and chelate treatments within a column indicate a significant difference $P<0.05$ according to LSD test.

Table 4.4 Lead accumulated amounts (mg plant^{-1}) in roots and shoots of signalgrass and atratum grown on lead contaminated soils with chelate treatments

Treatment (mmol kg^{-1} soil)	Signalgrass		Atratum	
	Root	Shoot	Root	Shoot
EDTA				
0	0.33 b	0.60 a	0.65 a	0.83 a
1.5	0.49 a	0.72 a	0.37 ab	0.49 b
2.5	0.43 a	0.70 a	0.56 ab	0.51 b
5	0.42 a	0.72 a	0.45 ab	0.27 b
10	0.24 b	0.56 a	0.21 b	0.31 b
Citric acid				
0	0.33 bc	0.60 ab	0.65 a	0.83 a
1.5	0.36 b	0.86 a	0.82 a	0.58 b
2.5	0.59 ab	0.58 ab	0.36 b	0.37 b
5	0.42 b	0.63 ab	0.37 b	0.42 b
10	0.77 a	0.50 b	0.34 b	0.43 b

a-c Mean values with different letters in the same species and chelate treatments within a column indicate a significant difference P<0.05 according to LSD test.

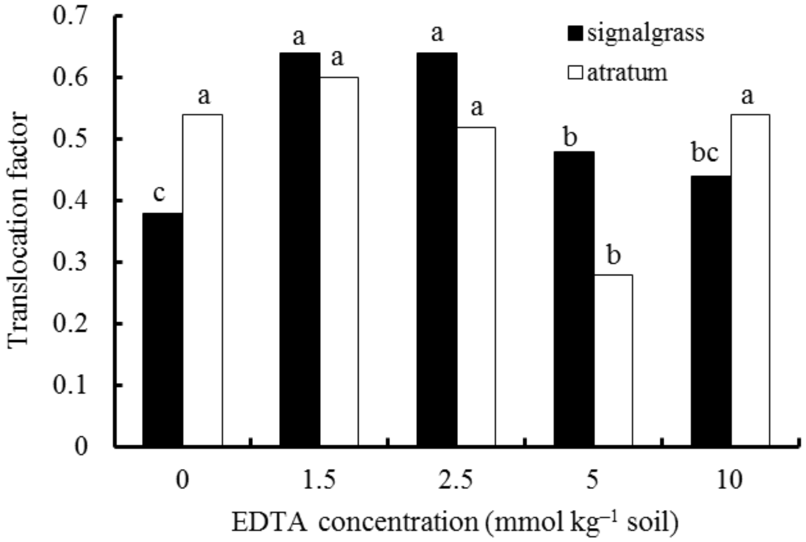

Figure 4.1 Effects of EDTA on translocation factor in signalgrass and atratum. Different letters in the same species indicate a significant difference at $P<0.05$ according to LSD test.

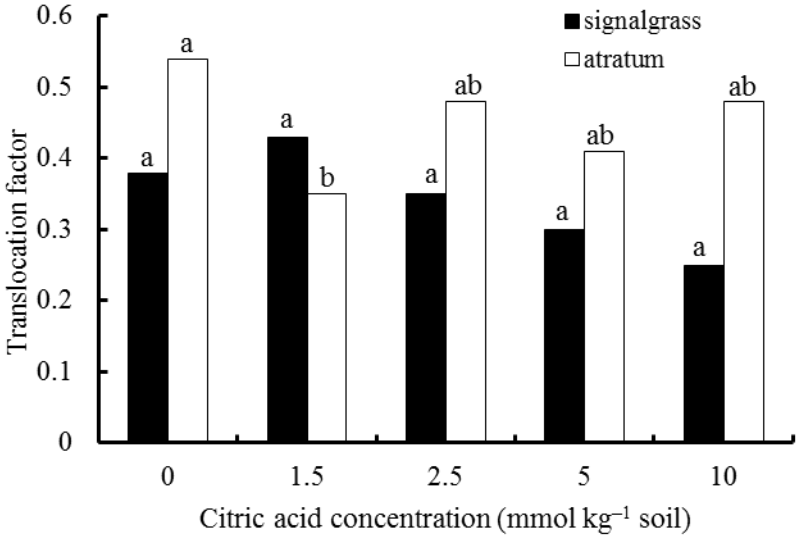

Figure 4.2 Effects of citric acid on translocation factor in signalgrass and atratum. Different letters in the same species indicate a significant difference at $P<0.05$ according to LSD test.

Discussion

Although both grasses grew apparently healthy on EDTA treated soils, at the end of the experiment, their dry matter was much lower than the control. On the other hand, higher concentrations of citric acid resulted in dry matter decrease of both grasses, probably due to the destruction of the physiological barrier by citric acid in roots which controls the uptake of solutes (Vassil *et al*. 1998). However, the root dry matter of signalgrass was higher in some EDTA and citric acid concentrations. Piechalak *et al*. (2003) explained that only lead nitrate addition caused the inhibition of root elongation growth and browning of roots, however, the addition of EDTA eliminated to a great degree the inhibition of root elongation growth, lower roots browning and resulted in a growing number of side roots. This result proved that the exogenous EDTA enhanced the endurance of root under lead stress and EDTA might act as a protective role against lead toxicity.

Though both EDTA and citric acid addition generally increased the shoot lead concentrations in signalgrass, EDTA (1.5, 2.5 and 5 mmol kg^{-1}) treated soil significantly increased the concentrations of lead in the shoots of signalgrass by 1.4, 1.5 and 1.3-fold, respectively, in comparison with the control (Table 4.3). The increase in lead concentration of signalgrass by the addition of EDTA was not as high as stated by Santos *et al*. (2006). Citric acid at the dose of 1.5 mmol kg^{-1} soil indicated the highest lead concentration in signalgrass among the citric acid amendments, and caused a 1.8-fold increase in shoot lead concentration, as compared to the control. Most of the increased lead uptake after the chelate treatments could be explained as an effect of enhanced lead solubility (Wu *et al*. 1999). It was reported that the concentration of lead in plant shoots correlated with the formation of lead-EDTA complex, either in solution or in a contaminated soil, suggesting that lead-EDTA was the major form of lead absorbed and translocated by the plant (Vassil *et al*. 1998; Epstein *et al*. 1999). EDTA and citric acid had no enhancing effect in the uptake of lead into the atratum, even they showed the decreasing effect of lead contents in roots and shoots (Table 4.3). Although EDTA is an efficient chelator of lead, the present results revealed that atratum roots did not possess a system for transporting EDTA metal complexes. Xu *et al*. (2007) reported that the roots of sorghum were inefficient in uptake of lead-EDTA complex. Athalye *et al*. (1995) explained that the stable lead-EDTA complexes in the soils were not in a plant-available form, even they decrease lead uptake. The lowering of lead concentration in roots treated with citric acid was probably related to the forms of lead in the solution, which is in agreement with the results of Chen *et al*. (2003), who also reported that citric acid could alleviate lead toxicity in radish, and decrease the lead uptake by the roots. Based on the present data, atratum could uptake lead even without the application of chelates. Interestingly, this effect was different between the two investigated plant species. This was probably due to the differential degradation of roots and associated release of labile metal fractions in the soil. This study indicated that the effectiveness in increasing the lead uptake by the application of chelating agents depends not only on the chelating agent but also on the plant species.

The effect of EDTA on the TF was higher than that of citric acid in both grasses. Luo *et al*. (2005) has proved that the application of EDTA significantly increased the shoot to root ratios of the concentrations of Cu, Pb, Zn, and Cd in corn (*Zea mays* L. cv. Nongda 108) and bean (*Phaseolus vulgaris* L. white bean). After the addition of citric acid, the decreased values of TF showed that plants stored higher amounts of lead in their roots, and this fact might have a negative effect on metal translocation from the roots to the shoots.

The potential of phytoextraction relies not only on the high metal concentration in shoots but also on the shoot dry matter (Nascimento and Xing 2006). Thus, higher dry matter can compensate for lower metal-concentrated shoots. Although increasing doses of EDTA resulted in increasing shoot lead concentrations and accumulated amounts in signalgrass (Tables 4.3 and 4.4), there was not significantly different because of slightly reduction of plant shoot dry matter (Table 4.2). Similarly, a dose of 1.5 mmol kg^{-1} citric acid leading to a maximum amount of lead accumulation could not be significantly determined in signalgrass. A significant decrease in lead accumulated amount of atratum was noted when soil was treated with increasing amounts of EDTA and citric acid. These results indicated that both EDTA and citric acid were ineffective as an amendment to enhance the lead phytoextraction by atratum. In any case, signalgrass was more effective than atratum regarding to the additions of EDTA and citric acid.

Conclusion

The high persistence of lead-EDTA complexes in the soil, the toxicity of free EDTA on plants and their leaching into the groundwater poses a great environmental risk (Lombi *et al.* 2001). Environmental concerns will require that the chelate addition be minimized. In the present study, signalgrass was able to grow in the presence of EDTA and citric acid without showing any phytotoxicity symptoms and could have the ability of metal tolerance. Addition of chelates in the range of 1.5 to 5 mmol kg^{-1} soil

EDTA and 1.5 mmol kg^{-1} soil citric acid was capable of increasing the lead uptake in the shoots of signalgrass. Conversely, the lower amounts of chelates yielded better results to lessen the leaching of metals in soils. In atratum, the control plants were more efficient in the uptake and translocation of lead than when EDTA and citric acid were added. The effectiveness of lead phytoextraction could be enhanced by the right combination of plant species and chelates. Signalgrass might be metal tolerant, showed comparative high dry matter while accumulating high concentrations of lead in their shoots and then could be suggested as a suitable candidate for chelate-induced phytoextraction of lead.

CHAPTER 5

Studies on animal performance, lead concentration in eggs, blood and feces of laying hens by feeding lead contaminated forage

Abstract

The present study with laying hens was designed to examine the effects of dietary forage lead levels on animal performance and lead concentration in blood, eggs (albumen and yolk) and feces during 5 weeks of feeding period. A total of 45 white leghorn layers were divided into 3 treatment groups: diet I contained only commercial layer feed, diet II and diet III included commercial feed with 7% each of low and high lead contaminated forage at the level of 8.94 and 91.47 mg kg^{-1} lead, respectively. There were no significant differences in live weight, egg production rate and average egg weight among all groups of laying hens. The dry matter digestibility in diet II and diet III showed a significant reduction compared to the control diet I. No measurable quantity of lead was found in the albumen of the eggs and blood of all treatment groups. The fecal lead concentration increased with an increase in lead levels in diets. Lead

concentrations in egg yolk for laying hens fed by diet I, diet II and diet III were 0.03, 0.06 and 0.25 mg kg^{-1}, respectively. Increased lead concentrations in feces and egg yolk were indicative of a greater degree of lead levels in the diets. However, the lead concentration in egg samples treated with 7 % low lead contaminated forage added diet II was within the permissible limit for lead during experiment and it showed the normal range as the control 4 weeks after experiment. Moreover, there were no significant negative impacts on the performance of laying hens treated with diet II groups. Then, available forage ingredients in poultry feeding could be eco-efficient to incorporate livestock and 7% low lead contaminated forage could be used as a safety level in the addition of poultry feed.

Introduction

Lead is one of the ubiquitous elements in the environment. Lead is found in the soil, plants and grains grown on contaminated soil, and at low levels in almost all living organisms (Doganoc 1996). Lead contaminated plants and soil are a major source of contamination to livestock (Brams and Anthony 1983). When the contaminated plants and feed grains are ingested by animals, dissolved lead may then be absorbed through the intestine wall into the blood stream, becoming deposited in soft tissues (Bakalli *et al.* 1995). Residual, unabsorbed lead is excreted into the feces.

Lead poisoning can occur in all domestic animals including horses, poultry and dogs (Khan *et al.* 2008). Lead produces acute and chronic poisoning and induces a broad range of physiological, biochemical and behavioral dysfunctions in animals. Poultry could take up heavy metal compounds from different sources and metal residues may concentrate in their eggs (Baykov *et al.* 1996; Demirezen and Uruc 2006; Nisianakis *et al.* 2009). Clinical signs of acute lead poisoning in chickens include muscle weakness, ataxia, and loss of appetite, followed by marked weight loss and eventual cessation of egg production. The effect of dietary lead exposure in chickens has been previously reported by several authors (Brams and Anthony 1983; Trampel *et* *al*. 2003; Yuan *et al*. 2013). There still has limited information on the study of dietary lead effect on laying hens by feeding lead contaminated forage. Davtyan and Manukyan (1987) reported that the fertility of hens increased when their diet included 14 % grass meal. Forages can have further positive effects when included in diets of monogastrics.

Therefore, chicken was used as a model animal and the addition of 7 % lead contaminated forage to the diet was better in the experiment according to preliminary trial period. Milling dried forages were used to reduce the volume substantially and animal selectivity. The present study with laying hens was designed to examine the effects of dietary forage lead levels on animal performance and lead concentration in blood, eggs (albumen and yolk) and feces.

Materials and methods

5.1 Experimental diets

Lead contaminated forage was cultivated from the greenhouse of the University of the Ryukyus, Okinawa. A commercial layer feed was used as the main portion of the diet. 7 % each of low and high lead contaminated forage were added to commercial layer feed. They were individually weighed using a 20 kg kitchen weighing scale and were thoroughly mixed together manually. Each experimental diet was separately prepared and properly kept in the storage plastic boxes until required for use. The control diet I contained only commercial layer feed, while diet II and diet III contained 7 % lead contaminated forage at the level of 8.94 and 91.47 mg kg^{-1}, respectively.

5.2 Animals and experimental design

The experiment was carried out using 58 weeks-old 45 laying hens (white leghorn). The animals were individually housed in cages and arranged to three groups with similar live weight. Each treatment group was replicated three times with 5 hens per replicate in a completely randomized design. Before the beginning of the experiment, animals were provided with a basal diet for 14 days adjustment period. The animals had ad libitum access to water and feed. Throughout the experiment, draining of remaining water, washing of the watering trough and supplying of fresh clean cool water, removing of remaining feed from the feeders and adding fresh feed were carried out on daily basis. The study was conducted at University of the Ryukyus, Okinawa, Japan for a period of 5 weeks between 26 June 2016 and 30 July 2016. Experimental plan and management of fowls was followed the guideline (Prime Minister’s Office 1970).

5.3 Performance and digestibility

Live weight measurements were determined prior to the start of the experiment and at the end of the experiment. Throughout the experiment, feed consumption, the number of eggs and egg weight were recorded every day. The egg production was expressed as an average hen-day production. Feed conversion ratio (FCR) was calculated as the ratio between feed intake and total weight of produced eggs.

The dry matter digestibility trial with the experimental diets was carried out by the method described by Takemasa (2001). The digestibility and fecal output trial was performed for 10 days at the end of the experiment. The fecal drops were collected from 5 hens per each replicate and conducted in the same time throughout the digestibility trial period. The excrement samples were oven-dried at 70°C for 48 hours and then were ground for chemical analyses.

5.4 Laboratory analyses

Samples of the experimental diets were analyzed according to the methods described by AOAC (1985). The proximate composition, metabolizable energy (ME) value was calculated by the method described by Fisher and Boorman (1986). The gross energy was determined by using Auto-calculating Bomb calorimeter (CA-4AJ, Shimadzu Co. Ltd., Japan). Eggs were thoroughly rinsed in distilled water and allowed to air-dry, and then separated into albumen and yolk. Each albumen and yolk was thoroughly blended before weighing for analysis, and the egg samples at 0.5 g were wet digested in concentrated nitric acid and 30 % hydrogen peroxide mixture (7:1, v/v) using a heat block digestion system. At the end of the feeding trials, blood samples were taken from the wing veins into labelled sterilized tubes containing an anticoagulant. Eggs from each experimental group were continually collected until 4 weeks after the experiment to determine the lead concentration in egg yolks.

Lead in the whole blood and dried forage samples was digested with concentrated nitric acid. The dried fecal samples at 0.5 g were digested in a mixture of concentrated nitric acid and 35 % hydrochloric acid (7:1, v/v) using a microwave laboratory system (Start D, Milestone General K.K. Co.Ltd., Kawasaki, Japan). The digested solution was filled up to 100 ml final volume with deionized water, and filtered through 0.45 µm sieve (Advantec, Tokyo, Japan). Then, concentrations of lead were analyzed using an inductively coupled plasma atomic emission spectroscopy (ICP-AES) (ICPE-9000, Shimadzu Co.Ltd., Kyoto, Japan). The lead concentration in the blood and egg contents was expressed on a wet-weight basis, whereas that in feces and forage was on a dry-weight basis.

5.5 Statistical analysis

The collected data were subjected to one-way analysis of variance using the General Linear Model (GLM) procedure of SPSS 16.0 (2007). Values were considered significantly different when P values were less than 0.05. However, lead levels in egg yolk were not normally distributed (Kolmogorov-Smirnov test statistic, $P<0.05$), so non-parametric statistics (Kruskal-Wallis test which is suited for small datasets with non-normal distributions) were used to explore differences among dietary treatments.

Results

The chemical composition of experimental diets was shown in Table 5.1. The crude protein and ash content were significantly increased in the control diet I ($P<0.05$).

The crude fiber and nitrogen free extract were significantly increased in diet II and diet III which contained 7 % low and high lead contaminated forage ($P<0.05$). The lead contents in the contaminated forage were 8.94 and 91.47 mg kg^{-1} DM, respectively. The dry matter digestibility in diet II and diet III showed a significant reduction compared with the control diet I ($P<0.05$) (Table 5.2).

The effects of dietary lead level on performance were presented in Table 5.3. At the end of feeding experiment, there was no significant difference in live weight among the three groups of chickens. The feed intake among the three treatment groups showed a significant difference ($P<0.05$), whereas the lowest and highest feed intake were found in diet II and diet III compared with the control. No significant differences were observed in egg production rate and average egg weight among all groups. However, the lowest egg production rate was recorded in the hens fed diet II. Feed conversion ratio was significantly higher in diet II compared with diet III. No mortality was observed throughout the experimental period.

The effects of dietary lead level on concentration of lead in albumen, yolk, blood and feces were shown in Table 5.4. No measurable quantity of lead was found in the albumen of the eggs. Lead concentration in yolks increased with increasing lead contents in diets. Similarly, fecal lead concentration increased with an increase in lead levels in diets. The blood lead levels in all laying hens of diet II and diet III were below detection limit.

The effects of dietary lead level on lead concentration in egg yolk during and after experiment were shown in Table 5.5. The egg yolk lead concentration in laying hens fed by diet I and diet II increased from first to third week after experiment and then showed the lower values in the fourth week. The lead concentrations in egg yolks of diet III were not detected after the experiment.

Table 5.1 Chemical composition of experimental diets

Parameters	Diet I (Control)	Diet II (Low)	Diet III (High)
Chemical composition, %			
Dry matter	89.12 a	89.84 a	90.07 a
Crude protein	25.39 a	20.99 b	22.06 b
Crude fiber	2.38 b	5.21 a	5.29 a
Ether extract	7.60 a	7.31 a	7.40 a
Ash	13.00 a	11.58 b	11.94 b
Nitrogen free extract	40.51 b	44.93 a	43.62 a
Calcium	3.12 a	3.20 a	3.12 a
GE (kcal kg^{-1})	4301.50 a	4346.60 a	4341.30 a
ME† (kcal kg^{-1})	2992.92 a	2963.58 a	2963.95 a
Lead (µg kg^{-1})	-	0.06 b	0.64 a

a-b Mean values with different letters on the same row are significantly different ($P<0.05$).

Diet I : Layer feed

Diet II : Layer feed with the addition of 7% low lead contaminated forage (8.94 mg kg^{-1} DM)

Diet III : Layer feed with the addition of 7% high lead contaminated forage (91.47 mg kg^{-1} DM)

†Metabolizable energy value was calculated using the method of 37 x %CP + 81 x %Fat + 35.5 x %NFE for poultry (Fisher and Boornan 1986).

Table 5.2 Effects of dietary lead level on fecal lead digestibility and metabolizable engery

Parameters	Diet I (Control)	Diet II (Low)	Diet III (High)
DM, %	22.05 a	20.30 a	21.36 a
GE (kcal kg^{-1})	3034.99 b	3266.67 a	3290.39 a
DM digestibility, %	75.55 a	72.06 b	72.04 b

a-b Mean values with different letters on the same row are significantly different ($P<0.05$).

Refer to Table 5.1 for the types of diet.

Table 5.3 Effects of dietary lead level on performance

Parameters	Diet I (Control)	Diet II (Low)	Diet III (High)
Initial weight (kg hen^{-1})	1.71 a	1.71 a	1.71 a
Final weight (kg hen^{-1})	1.72 a	1.66 a	1.71 a
Feed intake (g hen^{-1} day^{-1})	104.60 b	101.99 c	106.62 a
Egg production rate (%)	94.77 a	90.09 a	93.87 a
Average egg weight (g)	58.92 a	59.19 a	59.06 a
FCR (kg feed^{-1} kg egg)	1.78 ab	1.73 b	1.81 a
Mortality (%)	0	0	0

a-c Mean values with different letters on the same row are significantly different ($P<0.05$).

FCR: Feed conversion ratio

Refer to Table 5.1 for the types of diet.

Table 5.4 Effects of dietary lead level on concentration of lead in albumen, yolk, blood and feces

Items (mg kg^{-1}; wet basis)	Diet I (Control)	Diet II (Low)	Diet III (High)
Albumen	ND	ND	ND
Yolk‡	0.03 b	0.06 b	0.25 a
Blood	ND	ND	ND
Feces (mg kg^{-1}; DW)	30.68 c	33.14 b	44.93 a

a-c Mean values with different letters on the same row are significantly different ($P<0.05$).

ND – Not detected

‡ Non-parametric, Kruskal-Wallis test was used for small datasets with non-normal distributions.

Refer to Table 5.1 for the types of diet.

Table 5.5 Effects of dietary lead level on concentration of lead (mg kg^{-1}; wet basis) in egg yolk during and after experiment

	Diet I (Control)	Diet II (Low)	Diet III (High)	Kruskall-Wallis (X^2, P)
During experiment	0.03	0.06	0.25	14.78, $P<0.05$
Range	0.00–1.00	0.00–1.40	0.00–3.88	
After experiment				
First week	0.49	0.14	ND	5.21, $P<0.05$
Range	0.23–0.69	0.00–0.58		
Second week	0.26	0.88	ND	1.56, $P>0.05$
Range	0.00–1.06	0.00–5.28		
Third week	0.25	0.93	ND	0.74, $P>0.05$
Range	0.00–0.86	0.00–5.57		
Fourth week	0.05	0.09	ND	0.39, $P>0.05$
Range	0.00–0.28	0.00–0.42		

ND – Not detected

Refer to Table 5.1 for the types of diet.

Discussion

In general, animal performance was normal throughout the experiment. However, the average daily feed intake of hens consuming diet II which included 7 % low lead contaminated forage was slightly, but significantly ($P<0.05$) lower than it was for hens consuming control diet I without contaminated forage and diet III with 7 % high lead contaminated forage (Table 5.3). As a consequence, the diet II treated hens showed a reduction in final live weight at the end of the experiment. But egg production rate and average egg weight did not show any significant difference among the treatment groups. As this effect was not systematic, it could be attributed to temporary reduced performance of some animals. The absence of mortality among all the experimental hens showed that lead contents in the diets were not lethal doses to the animals. The decline in DM digestibility of diet groups treated with lead contaminated forage might probably be due to the presence of the increased amount of dietary fiber (Table 5.1).

Lead concentrations in albumen were non-detectable levels and were not influenced by the two diets containing 7% lead contaminated forage (Table 5.4). As expected, control animals showed a low-level background contamination of lead in the yolk. On the other hand, laying hens consuming diet II and diet III which contained 7 % low and high lead contaminated forage showed increasing lead concentration in egg yolk. Other studies have reported that little to no lead can be detected in the albumen, while the yolk contained much higher concentrations of lead (Mazliah *et al*. 1989; Hirai *et al*. 1991; Jeng *et al.* 1997; Trampel *et al*. 2003). According to the avian physiology, ova (egg yolk) are persistent before the formation of albumen layers in an egg (Sturkie and Mueller 1976). It might be lead could deposit more in egg yolk than in the albumen. Furthermore, metallothionein which shows a highly active defense of the embryo against lead II ions, was found the highest content in albumen (more than 12 μg g^{-1}) followed by yolk and kidney, and the lowest in liver (Hynek *et al*. 2012). Gagnon and Patel (2007) also implied that metallothionein had an important role as a tolerance mechanism against heavy metals toxicity. The blood lead concentrations were not detected in the study. Mahaffey and Michaelson (1980) indicated that the increased lead absorption was related to the calcium deficiency in diet. Calcium composition was almost similar in all experimental diets. It was noted that the increased lead concentrations in yolk were indicative of a greater degree of lead levels in the diets. Furthermore, lead residues varied depending on the exposure lead levels and specific organs.

The maximum permissible level of lead in poultry is 0.1 mg kg^{-1} (FAO/WHO 2002b; EC 2006) and 0.2 mg kg^{-1} (MHPRC 2005). The lead concentrations in egg yolk for laying hens fed by diet I, diet II and diet III during experiment were 0.03, 0.06 and 0.25 mg kg^{-1}, respectively. At the fourth week after experiment, the lead concentrations of egg yolk treated with diet I and diet II were 0.05 and 0.09 mg kg^{-1}, respectively. Lead residues in egg yolk were found to be of accepted level for the diet I and diet II treated groups.

The higher concentrations of lead in chicken feces suggested that the metal had passed directly through the digestive tracts or organs and been immediately excreted since feces might be an important elimination or avoidance route. Feces reflect short term exposure to lead, which normally undergoes limited bio-uptake and intestinal absorption, and consequently exhibits a high fecal excretion rate (Martínez-Haro *et al*. 2010). Therefore, increased dietary lead levels induced the increasing fecal lead concentrations in the experiment.

Conclusion

In conclusion, the consumption of dietary lead levels throughout the experimental period did not affect animal performance. The fecal lead concentrations were significantly higher in laying hens fed diets containing 7% lead contaminated forage. Accumulations of lead in the egg yolk greatly depended upon the administered dose. The lead concentration in egg samples treated with 7 % low lead contaminated forage added diet II was within the permissible limit for lead. There were not shown significantly negative impacts on the performance of laying hens treated with diet II groups and available forage ingredients in poultry feeding could be eco-efficient to incorporate livestock. Therefore, 7% low lead contaminated forage could be used as a safety level in the addition of poultry feed.

CHAPTER 6

General Discussion

Many researchers have studied a cost-effective, environmental friendly technique, by using green plants to eliminate heavy metals from the contaminated soils. Grasses have been more preferable in use for phyto-accumulation than shrubs or trees because of high growth rate, more adaptability to stress environment and high biomass. Most of the species were efficient to take up and translocate more than one metal from roots to shoots (Malik *et al.* 2010). The choice of phytoremediation technology to be employed for remediation of metal-contaminated sites depends on soil type, plant species, type of metal, degree and extent of contamination and environmental disturbance involved.

The first study investigated the phytoremediation abilities of some tropical pasture grasses using two types of soils (Chapter 2). Depending on different soil pH, the lead concentration in roots and shoots were varied among three tropical pasture grasses. In my present study, low soil pH (Kunigami-maji soil) had the higher uptake of lead in all experimental pasture grasses than high soil pH (Shimajiri-maji soil). It confirmed that pH seems to be a major element in controlling of lead mobilization. High soil pH may restrain the absorbability of the elements from the soil solution and translocation into plant tissues (Liu *et al*. 2005). Species retain metals in their roots and limit metal mobility from roots to shoots once absorbed by roots of plants (Cui *et al*. 2007). Even in the same Kunigami-maji soil, the efficiency of lead uptake was different among pasture grasses. Signalgrass and atratum could attain the highest lead concentration and accumulated amounts in their shoots without any marked negative impacts on their dry biomasses, and therefore could be useful for phytoextraction of lead in acidic Kunigami-maji soil. Napiergrass has revealed that it has the capacity to accumulate lead concentration in the roots and could be used for phytostabilization of lead pollution.

The second study was conducted to evaluate the lead uptake of three tropical pasture grasses by the effect of liming on the acidic Kunigami-maji soil (Chapter 3). Although the changes of soil pH significantly reduced the concentration of lead in three experimental grasses, it should be noted that the values for shoot lead concentration in napiergrass were the lowest among three grass species. Limed soils had little effect on lead accumulated amounts in signalgrass and atratum which could be used for phytoextraction. In phytostabilization, plants immobilize the metals in the rhizosphere thereby leaving them less bioavailable and less toxic to plants, animals and humans or retain the metals in the roots by restricting their translocation to above-ground parts (Wong 2003; Mendez and Maier 2008). Based on the results from Chapter 2 and Chapter 3, it can be concluded that napiergrass accumulates lead to high concentrations in its roots and restricts its translocation to shoots and therefore becomes a good candidate for phytostabilization of lead contamination in both acidic and alkaline soils. In being less expensive, less environmentally evasive and easy to implement, phytostabilization is considered to be more advantageous than other soil-remediation practices (Berti and Cunningham 2000). Moreover, above-ground part of plant tissues with low lead concentrations could be consumed by humans or animals. For more heavily contaminated soils, phytostabilization seems to be better at stabilizing the contaminated sites with tolerant plants in order to reduce the risk of erosion and leaching of these pollutants to water bodies. This observation is very beneficial to developing countries like Myanmar because napiergrass is widely distributed in many parts of the country.

The third study was continued to determine the chelate-assisted lead phytoextraction of signalgrass and atratum from contaminated soil (Chapter 4). The goal of phytoextraction is to maximize the metal accumulation in plants tissues and the mechanisms of internal tolerance are likely to be important. Tolerance to heavy metals is based on the sequestration of heavy metal ions in vacuoles, on binding them by appropriate ligands like organic acids, proteins and peptides and on the presence of enzymes that can function at high levels of metallic ions (Harborne 1989; Robinson *et al*. 1994). The choice of plant species and soil amendments are greatly important in the phytoextraction practices. Then, the amounts of added chelates had a great influence on shoot lead concentration and accumulation of each species. The present study clearly indicated that signalgrass had the better ability to uptake lead in the range of 1.5 to 5 mmol EDTA kg^{-1} treated soil and 1.5 mmol citric acid kg^{-1} treated soil than atratum and signalgrass could be used as chelate-assisted lead phytoextraction. Because of time being, phytoextraction will most likely be used in the medium to low levels of lead contaminated soils.

Phytoextraction deals with the absorption of toxic metals and metalloids by roots and their transportation to and accumulation in above-ground (harvestable) parts of plants resulting in reduced soil metal concentrations (Zhao and McGrath 2009; Ali *et al.* 2013). Harvested plant biomass from phytoextraction can either be disposed of as a hazardous material or, if economically feasible, used for metal recovery. Therefore, the utilization of lead contaminated forage as animal feed was studied in Chapter 5. Dietary inclusion of 7 % low and high lead contaminated forage did not show any negative impacts on the health of the laying hens throughout the experimental period (7 weeks including the preliminary period). With regard to the lead concentration, these trials showed that there were not detected the lead contents in albumen and blood of each of the three dietary treatments. The dietary treatment containing 7 % low lead contaminated forage found that the lead concentration in egg yolk was below the permissible limit for lead throughout the experiment and at the fourth week after experiment. Moreover, calcium and iron deficiencies in the diet could increase lead absorption in animals. Therefore, the minimum nutrient requirements in the diet formulation should be prepared when using the lead contaminated forage in the diet. Even small amounts of lead may affect the egg weight and egg production rate in laying hens, however, adding the grass meal into the diet increased the fertility of hens. Consequently, 7 % low level of lead contaminated forage could be safely added in the poultry feed for the utilization of phytoremediation by-products. Moreover, if harvested biomass contained high levels of lead, it could not be suitable for usage as animal feed in the present study.

The present study indicated that some tropical pasture grasses are applicable for the use of lead phytoremediation process in combination with suitable soil amendments in the areas from low to moderately lead contaminated soils and low level of lead contaminated biomass after harvest can be safely utilized in the addition of animal feed. However, there is a need for further research on the assessment of soil nutrients and other toxic pollutants present in soils.

CHAPTER 7

Summary

For the achievement of phytoremediation, selection of suitable plant species and appropriate soil amendments are greatly important. The ability to accumulate lead varies depending on plant species, type of soil, and amount of contaminants in the soil.

In Chapter 2, to investigate the phytoremediation abilities of tropical pasture grass species (signalgrass, napiergrass and atratum), two types of soils (Kunigami-maji soil and Shimajiri-maji soil) were contaminated with three levels of lead (0, 150 and 300 mg kg^{-1}). The dry biomass, lead concentration and the accumulated amounts were different among the plant species and between the soil types. The lead uptake by the examined grass species was more efficient in Kunigami-maji acid soil than in Shimajiri-maji alkaline soil. The difference in soil pH played a critical role in the bioavailability and uptake of lead by tropical pasture grasses. Signalgrass and atratum could uptake lead more than napiergrass and could be used for lead phytoremediation in acidic soil.

In Chapter 3, to examine the effect of liming to the acidic soil on lead uptake in three tropical pasture grasses, five levels of lime (0, 1, 2, 3 and 4 g kg^{-1} soil) was added to 150 mg kg^{-1} lead contaminated soil. Although an elevation of soil pH induced by liming resulted in a significant reduction of lead uptake in all experimental grasses, the effectiveness of liming varied with the pH values of soil and plant species. The adaptive soil pH level for each grass species might vary for optimal dry matter production and lead accumulated amounts. Napiergrasss maintained high lead concentrations in the roots and low lead concentrations in the shoots, and could be considered as a suitable candidate for phytostabilization of lead contamination. Signalgrass and atratum had the high lead accumulated amounts in shoots and could be useful for lead phytoextraction.

In Chapter 4, to evaluate whether two tropical pasture grasses (signalgrass and atratum) were applicable for the phytoextraction of lead, chelates (EDTA and citric acid) at the rates of 0, 1.5, 2.5, 5 and 10 mmol kg^{-1} soil were added to lead contaminated soils one week before harvesting. EDTA and citric acid addition increased the shoot lead concentration in signalgrass, however, EDTA showed more effective in shoot lead concentration of signalgrass than citric acid. On the other hand, atratum showed the highest lead concentration and accumulated amounts without the addition of chelates. These results indicated that the effectiveness in increasing the lead uptake by the application of chelating agents depends not only on the chelating agents but also on the plant species. Moreover, signalgrass was able to enhance lead uptake in the presence of lower amounts of chelates added that makes better results to lessen the leaching of metals in soils and could be suggested as a suitable candidate for chelate-induced lead phytoextraction.

In Chapter 5, to investigate the appropriate disposal of metal-enriched biomass after harvesting, a small percent of lead contaminated forage was formulated in the addition of commercial layer feed. Diet I contained only commercial layer feed and served as control. In diet II and diet III, 7 % of low and high lead contaminated forage containing at the level of 8.94 and 91.47 mg kg^{-1} respectively was added in the commercial feed. The addition of forage did not change the standard nutrient requirements of layer feed. With regard to the determination of lead concentration in egg (albumen and yolk), blood and feces, no measurable quantity of lead was found in egg albumen and blood. Lead concentration in egg yolk increased with increasing lead contents in diets, however, lead residues in egg yolks treated with diet II were found within the permissible level for lead during experiment and it showed the normal range as the control at the fourth week after experiment. Moreover, available forage ingredients in poultry feeding could be eco-efficient to incorporate livestock and therefore, 7 % low lead contaminated forage was a safe level in the addition of poultry feed.

In conclusion, high biomass tropical pasture grasses can be used as phytoremediation of lead in this study. It is clear from the present study that plant species, type of soil, the amount and type of soil amendments have a greatly influence on the lead uptake in pasture grasses. Soil pH is one of the major factors in controlling of lead phytoavailability and lower soil pH is more preferred to increase in lead uptake by plants. Selection of appropriate plant species and soil amendments is imperatively important to be successful for phytoremediation of lead. Napiergrass can be used for phytostabilization of lead in both alkaline and limed soils. Although both signalgrass and atratum could be used for phytoextraction of lead in lower soil pH, signalgrass indicated more effective in lead uptake because high dry matter of signalgrass compensate the moderate lead concentration in its shoot. As the utilization of lead-enriched harvested biomass, 7 % low lead contaminated forage containing 8.94 mg lead kg^{-1} can be added as a safe level to commercial feed without negative impacts on animal performance and within the permissible limit of lead in the eggs.

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