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Lead Accumulation and Isolation of Associated Rhizobacteria in Rice Grown in Lead Contaminated Soil

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Abstract

Lead accumulation in rice was studied using transmission electron microscope (TEM). The type of rhizobacteria associated with rice grown in lead contaminated soil was also examined. The results showed that rice plants accumulated lead in roots (5,735 mg/kg), stems and leaves (0.0820 mg/kg) and grains (0.0169 mg/kg). The concentration of lead in rice grains did not exceed the European Union Standard for lead in cereals (0.2 mg/kg) indicating that the seeds grown in lead contaminated soil were at acceptable levels. TEM studies were performed on root, stems and leaves of rice grown in control and lead contaminated soil after 120 days of planting. Most lead was accumulated in root cells in vacuoles of xylem parenchyma cell, lumen of xylem vessels, intercellular space of parenchyma in cortex and little was found in the cytoplasm. In stem cells, lead was found mostly in vacuoles of xylem vessels with very small amounts noted in the cell wall, intercellular space and cytoplasm. Leaf cell lead deposits appeared in vacuoles of xylem parenchyma cells with very small amounts noted in intercellular space and cell wall. Bacterial sampling of the rhizosphere identified four species of bacteria in lead contaminated soil associated with the roots of rice plants, namely *Pseudomonas chlororaphis* subsp. aureofaciens, *Microvirgula* sp., *Enterobacter* sp. and *Bacillus* sp. These results provide a baseline description of soil bacteria associated with the roots of rice plants growing in lead contaminated soil.

Keywords: Transmission electron microscope; Lead; Rice; Rhizobacteria

1. Introduction

Industry and agriculture in Thailand are co-evolving, often in the same area. Factories are established in the central region of the country where there are paddy fields posing challenges to food security. Rice, as the staple food of the Thai people, can be contaminated with heavy metals due to recurrent usage of metal-enriched agro-chemicals and the release of lead residuals from industrial activities. Heavy metals in agricultural lands may be transferred to humans and livestock through soil-plant-food interaction and may cause some serious health defects and abnormalities. Lead contaminated soil is an important environmental problem because of its toxic effect, its accumulation throughout the food chain, and the risk of groundwater contamination (Jebara *et al.,* 2015). Moreover, lead accumulation in rice grains can also cause human and livestock environmental quality issues and adverse health complications (Shraim, 2017). Some studies in humans showed that lead exposure may increase blood pressure, but the evidence is still inconclusive. Lead exposure may also cause anemia, resulting in a low number of red blood cells. Effects of high blood lead levels on children have been reported to reduce intelligence, cognitive development and functioning. Mitchell *et al.* (2012) found that SE Asian refugee children in the United States had permanent neurocognitive sequel and neurobehavioural toxicity from lead exposure.

Soil microorganisms may affect heavy metal availability by the processes of biosorption, bioaccumulation and solubilization (Marschner *et al.,* 1996). Microbes in the rhizospheric soil and in or attached to the roots of plants including rice can promote plants to accumulate extra heavy metals. Several studies showed the effect of heavy metal pollution on soil indices such as enzyme activity and microbial community activities and noted the changes of these indices following heavy metal bioreme diation. The main objectives of this study were to examine lead accumulation in the tissue of rice using TEM and to complete an initial isolation and identification of dominant rhizobacterial strains from the root area of rice grown in lead contaminated soil. The results of these studies will provide the basis for future investigations on the relationship between the dominant species of bacteria in the soil adjacent to the rice plant roots and lead uptake and resistance in rice.

2. Materials and Methods

2.1 Soil sampling and preparation

The soil used in this study was collected from Klity village of Kanchanaburi province, Thailand. Soil was collected at a depth of 0-20 cm at five points in the paddy field. The following procedures were used to characterize selected soil parameters as pH, OM, EC, available P and exchange K. Total soil lead was determined by analyzing the concentration of lead in soil following air-drying, crushing and sifting through a 0.5 mm sieve. Samples of 0.5 g of soil were digested with 5 mL of two concentrated acids mixed [$HClO_4$: HNO_3 (2:1 v/v)], in a block digester and filtered with Whatman paper No. 42. Samples were then adjusted to a volume of 50 mL with deionized water. The lead content of the acid extract was determined using flame and graphite atomic absorption spectrophotometry. All soil samples were air-dried, mixed, and placed in pots that were 30 cm in diameter. A total of 10 kg dry weight of soil was added to each pot. Thirty day old seedlings of *Oryza sativa* were transplanted in 5 replicate pots. Soil analyses were done at the beginning and end of the 120 day experiment.

2.2 Plant analysis

The control and treated rice plants were harvested after 120 days and washed with running tap water. Rice plants were divided into roots, stems and leaves and brown seeds. Roots, stems and leaves were cut into small pieces, while seeds were ground. Plant materials were dried for 2 days at 80°C. Approximately 0.2 g of a sample was dissolved in a 5 mL mixture of HNO_3 and $HClO_4$ at the ratio of 2:1. Samples were digested in a block digester at 200°C, filtered and adjusted to a volume of 50 mL with distilled water. The lead content of the acid extract was determined with a flame and graphite atomic absorption spectrophotometry.

2.3 Transmission electron microscope study

The root and aboveground samples from the control and lead-treated plants were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer and post-fixed in 2% osmium tetroxide. Samples were dehydrated through a graded series of ethanol concentrations (30, 50, 70, 80, 90, 95 and 100%) and embedded in Spurr's resin. Ultrathin sections were prepared by a grass knife and stained in 10% uranyl acetate and lead citrate, then examined under a TEM (Philips – TECNAI 20).

2.4 Isolation and identification of bacteria

Bacteria were isolated from soil samples by spread plate technique on lead acetate agar (proteose peptone, 20 g/L; Na_2HPO_4, 2 g/L; glucose, 10 g/L; agar 15g/L and adjusted pH 6.6) at lead acetate concentration 2% of 10 mL, and incubated at 37°C for 24-48 h. After incubation, bacterial colonies on the plate were observed for visual characteristics such as diameter, surface, edge and colony color. The cross-streak technique was performed on lead acetate agar and bacteria were incubated at 37°C for 24-48 h. A single colony was selected and checked for purity on lead acetate agar. Isolated lead-resistant bacteria were identified on the basis of 16s rRNA gene sequence. The genomic DNA was prepared by the extraction of the cell pellet using a GF-1 soil sample DNA extraction kit (Vivantis). Total extracted DNA were used for PCR-amplificaion of the 16S rRNA gene PCR using the universal primers (27F; 5'-AGAGTTTGATCCTGGCTCAG-3' and 1525R (5'-AAAGGAGGTGATCCAGCC-3') for the 16S rRNA gene specific region. The thermocycle program was as follows: denaturing at 94°C for 5 min, 30 amplification cycles of 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 2 min, with extended elongation at 72°C for 5 min. The PCR products 1,500 bp were analyzed by electrophoresis on agarose gel by an ABI PRISM 310 genetic analyzer. The nucleotide sequences of 16S rRNA gene of the unidentified bacteria were compared to those of 16S rRNA gene of the bacteria in the GenBank database. The experiment data was analyzed using the R-Base language, analysis of variance (ANOVA) for significant difference ($p<0.05$). Duncan's new multiple range test (DMRT) was used to determine significant difference ($p<0.05$).

3. Results and Discussion

3.1 Physio-chemical characteristics of contaminated soil

Table 1 presents lead contaminated soil characteristics as pH, EC, OM, available P and exchangeable K were 6.8, 0.4 dS/m, 3.7%, 1.0 mg/kg and 30.0 mg/kg respectively. The soil lead concentration before and after cultivation is also presented in Table 1. The treated plants grew well without evidence of lead toxicity indicating that the rice plants were tolerant of relatively high concentrations of lead. Different factors affect metal uptake and accumulation by plants. Soil pH values increased slightly from 6.6 on Day 0 to 6.8 on Day 120 possibly due to soil waterlogging. The soil organic matter and the EC decreased slightly over the 120 day experiment, from 4.5 to 3.7 and from 0.5 to 0.4 respectively. At the end of the experiment, available P and exchangeable K decreased, corresponding with nutrient uptake during rice growth and lead uptake as it was accumulated in the plant tissue.

3.2 Lead accumulation in rice

Lead concentration in different parts of rice were noted as roots > stems and leaves > grains (Table 2). The highest and lowest lead concentration of rice were seen in the roots and grains, respectively. Most lead was found in roots and less was transported to stems and leaves. Roots are the first organ exposed to lead and thus can be a major storage organ for lead or can play an intermediary role for exporting the lead ions from soil to the aboveground plant parts. Lane and Martin (1977) studied lead uptake in plants and have demonstrated that roots have an ability to take up significant qualities of lead whilst simultaneously greatly restricting its translocation to have aboveground parts. The limit transport of lead from roots to other organs is the result of a barrier caused by the root endodermis (Sharma and Dubey, 2005). At the root surface lead binds to carboxyl groups of mucilage uronic acids. Mucilage binding restricts metal uptake into the root and establishes an important barrier protecting the

Table 1. Physical and chemical characteristics of lead-contaminated soil at the end of the experiment (120 days)

Parameters	Analysis result	
	Day 0	Day 120
Potential of hydrogen ion: pH[1/]	6.6	6.8
Electric conductivity: EC[2/] (dS/m)	0.5	0.4
Organic matter: OM[3/] (%)	4.5	3.7
Available phosphorous: Avai.P[4/] (mg/kg)	4.0	1.0
Exchangeable potassium: Exch.K[5/] (mg/kg)	117.0	30.0
Total Pb[6/] (mg/kg)	15,300	10,910

[1/] 1:1 H_2O
[2/] 1:5 H_2O
[3/] Walkley and Black method
[4/] Bray II extraction
[5/] 1NH_4OAc pH
[6/] In-house method based on EPA Method 6010B

root system. Some of the bound metal is released when mucilage is biodegraded (Morel *et al.,* 1986). Panich-Pat and Srinives (2009) studied partitioning of lead accumulation in rice plants and found that rice plants accumulated lead in the respective order from high to low in roots, stems and leaves, bran, and husk. The amount of lead that is taken up by the plants can be measured and reported as "the transfer factor" (TF) or "the bioconcentration factor" (BCF) which differs with plant species and soil type (Bi *et al.,* 2010). Our study indicates that after uptake by the roots, lead moved to aerial parts of the rice plant. TF and BCF were $0.17x10^{-4}$ and 0.53, respectively. Although we noted acceptable levels of lead in rice grains, lead deposited in stems and leaves and bran might enter the food chain as cattle feeds, mulch in vegetable production, and release to soil and water after plant material decomposition. The extent of these environmental risks requires further investigation. Rice plants were obviously not suitable for use in phytoremediation applications in cleaning up lead contaminated areas. The maximum limit in the European Union established for lead in cereals is 0.2mg/kg and the maximum content for lead in animal feed is 30 mg/kg DW. In our pot experiments, the lowest lead concentration was recorded in grains (0.0169 mg/kg DW), which was well below the maximum content of the European Union Standard for lead in cereal for human consumption.

3.3 Transmission electron microscope study

The samples from both the control and treatments with the highest lead accumulation were examined with TEM (Philips–TECNAI 20). Root samples from the treatments with the highest lead accumulation had most of the lead granules appearing as deposits in vacuoles of xylem parenchyma cell (Figure 1C2, 1D2, 1F1), lumen of xylem vessels (Figure 1C1, 1D1), intercellular space of parenchyma in cortex (Fig. 1B1, 1E1). Insignificant deposits of lead were found in the cytoplasm (Figure 1B2). The normal cells without lead deposition are shown in (Figure 1A). Lead was described as a toxic element whose transport was hampered by immobilization within cell walls, precipitation of insoluble lead salts in intercellular spaces, accumulation in plasma membranes or sequestration in the vacuoles (Krzesłowska *et al.,* 2016). Panich-Pat and Srinives (2009) used

Table 2. Mean lead concentrations in organs of rice

Organs of rice	Lead concentrations (mg/kg-1DW)	
	Day 0	Day 120
Roots	$0.0507 \pm 0.0534^{1/b}$	$5{,}735.00 \pm 562.44^{1/a}$
Stems and leaves	$0.0058 \pm 0.0020^{1/b}$	$0.082 \pm 0.0146^{1/a}$
Grains	0.0136 ± 0.0047^{a}	0.0169 ± 0.0011^{a}

1/ Lead concentrations among different treatments were significantly different at $p<0.05$

a, b, c – Value in the same column followed by the same letters are not significantly different according to DMRT ($p<0.05$). Results represent means of 5 replicates ±SD

transmission electron microscopy and studied the partitioning of lead accumulation in rice plants and found presumptive lead deposition in vacuoles, around the endoplasmic reticulum and cytoplasm. For root metal uptake, generally the part of metal found in the soil solution is first adsorbed onto the root surfaces, followed by their binding to polysaccharides of the rhizodermal cell surface or carboxyl groups of mucilage uronic acid (Pourrut *et al.,* 2011). After adsorption on roots surface, heavy metals penetrate the roots passively and diffuse through translocating water streams. Metal movement within plant from roots to the aerial parts is via xylem loading, which is assisted by unidentified transport processes, and occurs via symplastic or apoplastic transport. Metals are transported with different chelates (Saebø *et al.,* 2012), and the transportation is generally governed by plant transpiration. The behavior of lead in soil and uptake by plants is controlled by its speciation and by the soil pH, soil particle size, cation-exchange capacity, root surface area, root exudation, and degree of mycorrhizal transpiration. After uptake, lead primarily accumulates in root cells, because of the blockage by Casparian strips within the endodermis, sequestration in the vacuoles of rhizodermal and cortical cells by the formation of complexes and immobilization by negatively charged pectins within the cell wall, binding by phytochelatins, glutathione, and amino acids and precipitation of insoluble lead salts in intercellular spaces (Kopittke *et al.,* 2007).

Rice plants accumulated lead in the stems which appeared to be deposited as lead granules in vacuoles of xylem vessels (Figure 2B2, 2C1). Insignificant amounts of lead were found in the cell wall (Figure 2B1), intercellular space (Fig. 2B3) and cytoplasm (Figure 2C2). Leaf cells were found in vacuoles of xylem parenchyma cells (Figure 3B1, 3C3) and little was found in intercellular space (Figure 3C1) and cell wall (Figure 3C2). Most lead granules noted in these experiments accumulated in vacuoles of the root, stem and leaf cells. Vacuoles are the largest storage pool in rice plants and protect the cell from the toxic effects of lead by sequestering the lead from the cytoplasm. Metal translocation to shoots is a crucial biochemical process (Tangahu *et al.,* 2011). Lead levels in different plant parts of rice followed the order: root > shoot >grain. Ultrastructural studies have revealed that variable amounts of lead deposits were present mainly in the intercellular space, cell wall and vacuole, whereas small deposits of this metal were seen in the endoplasmic

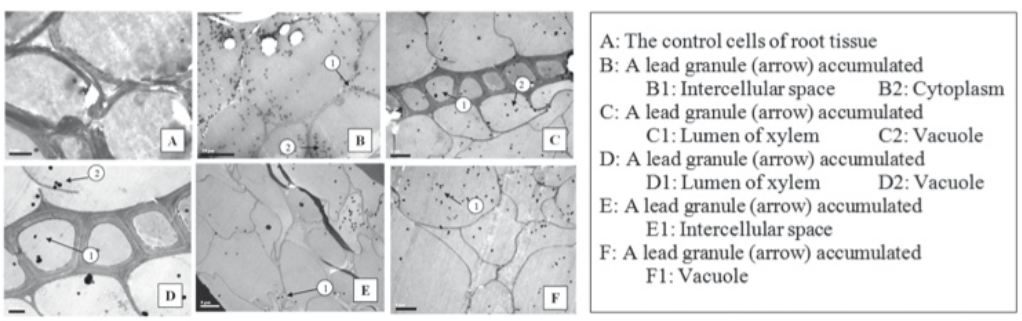

Figure 1. Transmission electron micrographs of root tissue in rice plant (*Oryza sativa* L.)

reticulum. The cell wall and vacuole together account for about 96% of absorbed lead (Wierzbicka and Antosiewicz, 1993). The fact that lead was found in the endoplasmic reticulum was apparently related to metal secretion of the cell surface into the vacuole. A small quantity of lead reached nuclei, chloroplasts and mitochondria and exerted its toxic effects on these organelles. Sometimes, particularly in close proximity to the plasmodesmata, the larger lead particles appear to occupy much of the volume of the cell wall. Lead retention in the roots is based on binding of lead to ion exchangeable sites on the cell wall and extracellular precipitation, mainly in the form of lead carbonate deposited in the cell wall. After being taken up by roots, the localization of lead is greater in roots than in other parts of the plants. Lead binds strongly to the carboxyl groups of the carbohydrates galacturonic acid and glucuronic acid in the cell wall, which restricts its transportation via apoplast (Rudakova *et al.,* 1988). In general dicots accumulated significantly higher amounts of lead in the roots than monocots (Huang and Cunningham, 1996). Lead transported from the soil to the root cells had to cross the root cell plasma membrane. One possible transport pathway of lead across the plasma membrane (PM) appeared to be through PM cation channels, such as Ca channels. A voltage gated Ca channel in the root cell PM has been characterized using right side out PM vesicles isolated from roots of wheat and corn plants (Huang *et al.,*1994). Huang and Cunningham (1996) found that lead significantly inhibited voltage gated Ca channels activity in the PM of wheat roots. The inhibition of the Ca channel by lead could arise from lead blockage of the channel or due to competitive transport of lead through the Ca channels.

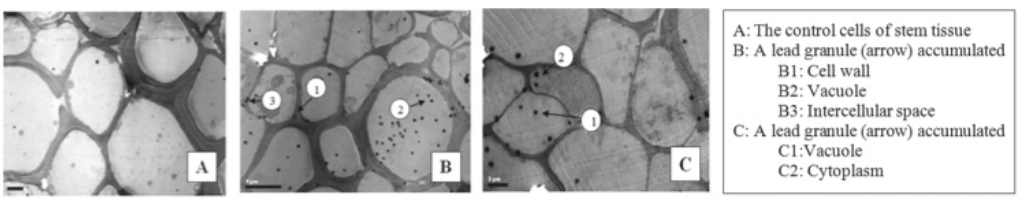

Figure 2. Transmission electron micrographs of stem tissue in rice plant *(Oryza sativa L.)*

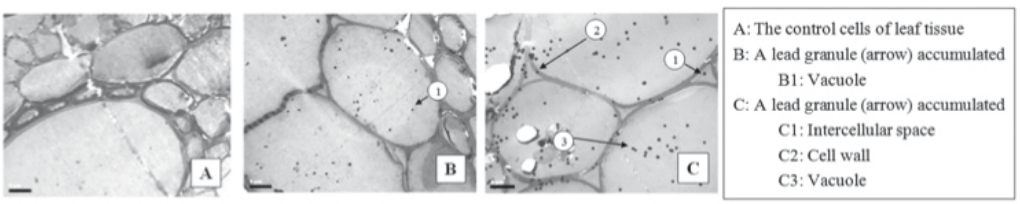

Figure 3. Transmission electron micrographs of leaf tissue in rice plant *(Oryza sativa L.)*

Table 3. Partial 16S rRNA sequences derived GenBank deposits

Code number	GenBank acc.no	Best match with	% Identity
RV2	AB680101.1	*Pseudomonas chlororaphis* subsp. aureofaciens	97%
RV4	KR029290.1	*Microvirgula* sp.	98%
TK1	JX847614.1	*Bacillus* sp. BDU13	97%
TK3	KF582906.1	*Enterobacter* sp. 2011SOCNI48	97%

3.4 Identification of rhizobacteria

The results showed four isolated resistant bacteria with lead concentrations as RV2, RV4, TK1 and TK3 (Table 3).

Identification of bacterial strain was done based on analysis of 16S rRNA gene sequence. The expected size of the PCR product was 1,500 bp (Figure 4). PCR products were sequenced by an ABI PRISM 310 Genetic Analyzer with a BigDye Termimator (version 3.0). The obtained sequences were compared to 16S rRNA gene sequence in the GenBank database using the BlastN program to retrieve sequence similarity and bacterial identification (Table 3). Bacterial isolates showed high identity 98% similarity with *Microvirgula* sp. and bacterial isolates showed identity 97% as *Pseudomonas chlororaphis* subsp. aureofaciens, *Enterobacter* sp. 2011SOCNI48 and *Bacillus* sp. BDU13. Some studies showed microorganisms that live on rhizosphere have the potential to increase the rate of plant growth and to replace heavy metals in the form that plants can be absorbed through the roots (Glick, 2010). Wyszkowska *et al.* (2008) studied the effects of bacteria in heavy metal-contaminated soil and showed that *Pseudomonas* sp. was resistant to lead despite using a transporter protein and phosphates. Kumar *et al.,* (2010) studied the ability of four acclimated microorganisms to accumulate heavy metals and concluded that *Pseudomonas* sp. and *Bacillus* sp. could absorb high lead concentrations. Soil microorganisms interact with plants in many different ways to reduce metal ion toxicity and enhance metal ion absorption by plants. In addition soil microorganisms may affect heavy metal availability by the process of biosorption, bioaccumulation and solubilization (Marschner *et al.,* 1996).

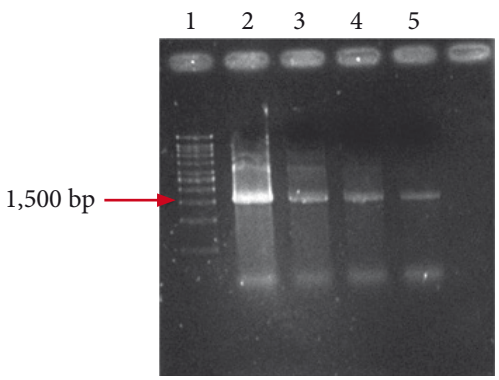

Figure 4. Representative of the PCR products of 16S rRNA gene primer that amplified around 1,500 bp. Front lane is DNA marker; Lanes 2-5 are representative of amplified 16S rRNA genes from bacterial strains

4. Conclusions

Rice plant accumulated lead in the respective order from high to low in roots, stems and leaves and grains. The concentration of lead in rice grain did not exceed the maximum acceptable content for lead in cereal standard. Transmission electron microscopic studies were performed on roots, stems and leaves of rice grown in control and lead contaminated soil on 120 days after planting. Most lead was accumulated in root cells in vacuoles of xylem parenchyma cell, lumen of xylem vessels, intercellular space of parenchyma in cortex and little was found in the cytoplasm. In stem cells were found in vacuoles of xylem vessels and little was found in the cell wall, intercellular space and cytoplasm. Leaf cells were found in vacuoles of xylem parenchyma cells and little was found in intercellular space and cell wall. This research also identified four species of bacteria associated with the roots of rice plants growing in lead contaminated soils., namely *Pseudomonas chlororaphis* subsp. aureofaciens, *Microvirgula* sp, *Enterobacter* sp. and *Bacillus* sp. The initial identification of the bacteria reported here can lead to future detailed studies of their specific role on the growth and uptake of lead in rice plants.

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