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Original Research

Lead Accumulation and Isolation of Rhizobacteria from Maize Grown in Contaminated Soil

Thitapa Keawsringam[1], Jintanart Wongchawalit[2], Thanawan Panich-Pat[1]*

[1]A Research Cluster of Environmental Science and Technology, Department of Science,
Faculty of Liberal Arts and Science,
[2]Department of Microbiology, Faculty of Liberal Arts and Science,
Kasetsart University, Kamphaengsaen Campus, Nakhon Pathom 73140, Thailand

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Abstract

This research aimed to study lead accumulation and the type of rhizobacteria associated with maize grown in the lead-contaminated area of Klity village, Kanchanaburi Province, Thailand. The results showed that lead concentrations in different tissues were roots > shoots > grains. The highest lead concentration was recorded on day 120 (54.31, 110.67 and 4.79 $mg \cdot kg^{-1}$ in shoots, roots, and grains, respectively). The lowest lead concentration was recorded on day 40 (27.80 and 71.90 $mg \cdot kg^{-1}$ in shoots and roots, respectively) with no detectable lead in the grain. Results indicate that lead concentration in grains on day 120 of the experiment exceeded the European Union Standard (0.2 $mg \cdot kg^{-1}$), which might not be safe for human consumption but did not exceed the standard as animal feed (30 $mg \cdot kg^{-1}$). This research found four species of bacteria that could grow in soil at a lead concentration exceeding 3,600 $mg \cdot kg^{-1}$, namely *Bacillus* sp. B26, *Pseudomonas* sp. S169, *Pseudomonas putida* strain RW10S2, and *Bacillus subtilis* strain SM10.

Keywords: lead, maize, rhizobacteria, Kanchanaburi province, soil remediation

Introduction

Heavy metal-contaminated soil is an important environmental problem because it results in accumulation throughout the food chain and the risk of groundwater contamination. Among the heavy metals, lead (Pb) is a potential pollutant that readily accumulates in soil [1]. In Thailand, the problems of lead-contaminated soil at Klity village, Thong Pha Phum District, Kanchanaburi Province, were first reported in 1998. The cause of soil contamination was untreated wastewater from mine ore dressing discharged into Klity Creek and distributed into the soil [2].

Maize is a familiar agricultural crop that is widely adapted and could be cultivated easily. It has greater dry mass than many heavy metal hyperaccumulating plants, such as *Thlaspi caerulescens* and *Arabidopsis halleri*. Maize could accumulate lead when grown in contaminated soil [3]. Several researchers have indicated that maize absorbs lead from soil and accumulates it in roots, shoots, leaves, and grains [4, 5].

The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, supports large active groups of bacteria [6]. Bacteria in soil are sensitive to the high concentrations of heavy metal and unnecessary heavy metals at low concentration. Bacteria adapt to the heavy-metal polluted soil by changing intrinsic biochemical, physiological, and structural properties and genes. Therefore, microbes that survive in polluted soil show higher resistance to heavy metals as compared to populations in non-contaminated sites [7]. The purpose of this study was to measure lead accumulation in roots, shoots, leaves, and grains of maize in Klity fields and to isolate rhizobacteria strains from the root area of maize grown in lead-contaminated soil.

*e-mail: g4237508@yahoo.com

Table 1. Physical and chemical characteristics of lead-contaminated soil.

pH	ECe (dS/m)	OM (%)	Total N (%)	C/N ratio	Avai P $mg \cdot kg^{-1}$	Avai K $mg \cdot kg^{-1}$	Lead (before cultivation)	Lead (after cultivation)
7.3	2.49	7.82	0.30	13.36:01	66.97	142.97	3,641.65	3,214.8

Materials and Methods

Sample Collection

The experiment was conducted in the maize field at Klity Village, Thong Pha Phum District, Kanchanaburi Province, Thailand. Soil and maize samples were collected in 400 m^2 of the maize field. Soil samples (1 kg used) were collected (depth 0-20 cm) from five locations in the field. All soil samples were mixed and used in soil characteristic and lead concentration analysis. Maize was harvested after 40, 70, and 120 days. One hundred maturing maize plants were chosen at random for analysis.

Lead Analysis

The soil samples were air dried at room temperature for one week. Dried samples were homogenized in a mortar, sieved through a 2 mm mesh, and analyzed for lead content. The plant samples were dried at ambient temperature for 24 h and then dried again in a hot air oven at approximately 105ºC for 48 h. Dried plant samples were homogenized in a blender, sieved through a 1 mm mesh, and analyzed for lead concentration. 0.5 g of plant or soil sample was digested with 15 ml mixture of HNO_3 (65%) and $HClO_4$ (70%) at a ratio of 1:2, v/v. All 0.5 g maize samples were digested with a 15 ml mixture of HNO_3 (65%) and $HClO_4$ (70%) at the ratio of 2:1, v/v, in a block digester and filtered with Whatman paper No. 42, and finally diluted to 25 ml with deionized water. Lead concentrations of all samples were determined by flame atomic absorption spectrophotometry (Spectra AA 200).

Isolation 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; agra, 15 g/l and adjusted pH to 6.6) at lead concentration of 60 $mg \cdot l^{-1}$, and incubated at 30ºC for 4-5 days. After incubation, bacterial colonies on the plate were observed for visual characteristics such as colony color, diameter, edge, and surface. The cross-streak technique was performed on lead acetate agar and bacteria were incubated at 30ºC for 4-5 days. A single colony was selected and checked for purity on lead acetate agar. Lead-resistant bacterial colonies were purified on media. To isolate strains with high lead tolerance, successive enrichments were carried out by gradually increasing the concentration of lead in the medium from 100 to 3,600 $mg \cdot l^{-1}$. The bacterial culture showing lead resistance was selected for further study.

Identification of Bacteria

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-amplification of the 16S rRNA gene PCR using the universal primers (27F; 5'-AGAGTTTGATCCTGGCTCAG-3' and 1512R (5'AAAGGAGGTGATCCAGCC-3') for the 16S rRNA gene specific region. The thermocycle program was as follows: denaturing at 94ºC for 5 min, 30 amplification cycles of 94ºC for 1 min, annealing at 60ºC for 1 min, and elongation at 72ºC for 2 min, with extended elongation at 72ºC 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.

Statistical Analysis

Data analysis was performed using ANOVA and Duncan tests. All statistical analysis was done using SPSS software version 16 with significance based on 0.05 in most cases.

Results and Discussion

Physio-Chemical Characteristics of Contaminated Soil

Lead-contaminated soil has pH 7.3, organic matter content of 2.49, variable nutrient contents with total N 0.30%, C/N ratio 13.36:01, high available P 66.97 $mg \cdot kg^{-1}$, and highly available K 142.97 $mg \cdot kg^{-1}$. The total lead concentrations in soil before and after cultivation were 3,641.65 and 3,214.8 $mg \cdot kg^{-1}$, respectively (Table 1), which exceeded the Thai standard level (400 $mg \cdot kg^{-1}$).

Lead Accumulation in Maize

Lead concentrations in different tissues of maize were roots > shoots > grains (Table 2). The highest lead concentration of maize was recorded on day 120 (54.31, 110.67, and 4.79 $mg \cdot kg^{-1}$ in shoots, roots, and grains, respectively). The lowest lead concentration was recorded on day 40 (27.80 and 71.90 $mg \cdot kg^{-1}$ in shoots and roots, respectively). The results were consistent with these of Sekara et al. [8], who studied lead accumulation and distribution in the

Table 2. Mean lead concentrations in maize from field experiments (results represent means of 100 replicates±SD, mg·kg^{-1}).

Day of cultivation	Lead concentration (mg·kg^{-1})		
	Shoots	Roots	Grains
40	27.80±14.64 [a]	71.90±35.25 [a]	Not detected
70	43.72±19.20 [b]	97.99±58.16 [b]	1.24±0.14 [a]
120	54.31±27.38 [c]	110.67±76.74 [c]	4.79±4.64 [a]

[a, b, c] – Value in the same column followed by the same letters are not significantly different according to DMRT ($p \leq 0.05$).

Table 3. Morphology of four isolated bacterial colonies.

Strain	Characteristics of colony on lead acetate agar	Gram
MKT 3 2.2 E	Circular shape, entire margin, yellow colony, smooth surface	+
MKT 4 2.1 G	Circular shape, entire margin, smooth surface, opaque optical	-
MKT 5 1.1 D	Circular shape and flat elevation, entire margin, translucent optical	-
MKT 5 2.1 A	Ellipsoid shape, flat elevation, lobate margin, opaque optical	+

organs of nine crop plants. They found lead in roots, stems, leaves, and grains with the measurement accumulation in roots>shoots and leaves >grains. The lowest lead accumulation in grains in comparison to other organs is due to the control of uptake and translocation of lead from the soil by plants [9]. Mojiri [10] also showed higher accumulation of lead in roots than in shoots of maize.

The maximum limit in the European Union established for lead in cereals is 0.2 mg·kg^{-1} fresh weight [11], but the maximum content for lead in animal feed is 30 mg·kg^{-1} dry weight [12]. In the field experiment, the highest lead concentration was recorded in grains on day 120 (4.79 mg·kg^{-1}), which exceeded the European Union Standard for consumption but did not exceed the maximum content for lead in animal feed.

A total of 21 isolates of rhizobacteria were able to tolerate lead at different concentrations (100-3,600 mg·l^{-1}). The results showed four isolated resistant bacteria with high lead concentration as MKT 3 2.2 E, MKT 4 2.1 G, MKT 5 1.1 D, and MKT 5 2.1 A. (Table 3).

Identification of Rhizobacteria

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 (Fig. 1). 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 4).

Table 4. Partial 16S rRNA sequences derived GenBank deposits.

Code number	GenBank acc. no	Best match with	% Identity
MKT 3 2.2 E	JX941521.1	*Bacillus* sp. B26 (2012)	99%
MKT 4 2.1 G	KC898248.1	*Pseudomonas* sp. S169	99%
MKT5 1.1 D	JN982334.1	*Pseudomonas putida* strain RW10S2	99%
MKT 5 2.1 A	JQ809795.1	*Bacillus subtilis* strain SM10	99%

Four bacterial isolates showed high identity, 99% similarity with *Bacillus* sp. B26 (2012), *Pseudomonas* sp. S169, *Pseudomonas putida* strain RW10S2, and *Bacillus subtilis* strain SM10. This finding was similar to that of Ashraf [13], who studied the effects of heavy metals on soil microorganisms. Lead resistance was found in *Bacillus* sp. and *Pseudomonas* sp. Wyszkowska et al. [14] 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 [15]. Soil microorganisms interact with plants in many different ways to reduce metal ion toxicity and enhance metal ion absorption by plants [16]. Likewise, Kumar et al. [17] studied the ability of four acclimated microorganisms to accumulate heavy metals and concluded that *Pseudomonas* sp. and *Bacillus* sp. could absorb high lead concentrations.

Conclusions

The lead concentration of grains on day 120 of the experiment exceeded the European Union Standard (0.2 mg·kg^{-1}), which might not be safe for human consumption but did not exceed the standard as animal feed

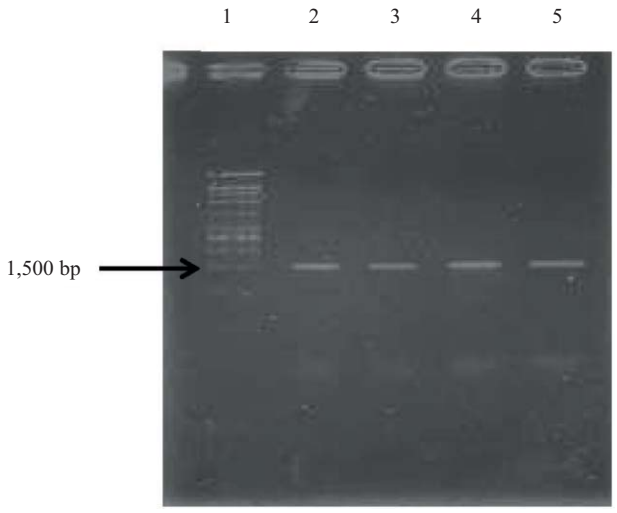

Fig. 1. Representative of the PCR products of 16S rRNA gene primer that amplified around 1,500 bp. Front lane is DNA marker; Lanes 2-5 are representative of amplified 16S rRNA genes from bacterial strains.

(30 mg·kg^{-1}). Moreover, this research found four species of bacteria that could grow in soil with lead concentrations exceeding 3,600 mg·kg^{-1}, namely *Bacillus* sp. B26, *Pseudomonas* sp. S169, *Pseudomonas putida* strain RW10S2, and *Bacillus subtilis* strain SM10.

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