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Article

Rhizosphere metagenome analysis and wet-lab approach to derive optimal strategy for lead remediation *in situ*

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SUMMARY

The Environmental Protection Agency (EPA) reports a significant number of heavy metal-contaminated sites across the United States. To address this public health concern, rhizoremediation using microbes has emerged as a promising solution, considering the limitations and inefficiencies of current approaches. We hypothesized that if *Pseudomonas fluorescens*, *Burkholderia vietnamiensis*, and *Rhizobium leguminosarum* are cultivated in the rhizospheres (area around the plant roots) of *Brassica juncea*, *Oryza sativa*, or *Pisum sativum* in lead-contaminated soil, then a significant reduction in soil-lead content should be observed. To determine the most effective soil microbe, we obtained raw sequences of 16s rRNA from 96 soil samples from the National Center for Biotechnology Information and processed the sequences through Qiime2 to examine bacterial taxonomy in lead-contaminated and uncontaminated rhizospheres. A combination of soil microbes – *P. fluorescens*, *B. vietnamiensis*, and *R. leguminosarum* – were inoculated in the rhizosphere of *B. juncea*, *O. sativa*, and *P. sativum* in soil contaminated with 500 parts per million (ppm) of lead. Soil lead content was measured at various stages of plant growth. After four weeks, we noticed a 70% decline in the lead content with *P. fluorescens-B. juncea* combination and a 90% decline with *P. fluorescens-R. leguminosarum-B. vietnamiensis* triple combination with *B. juncea*. Chlorophyll content analysis of the dried leaves of plant groups showed similar optical density to control leaves, indicating that lead decontamination in the soil did not negatively affect plant health. Therefore, rhizoremediation is an effective bioremediation strategy and may increase crop productivity by converting nonarable lands into arable lands.

INTRODUCTION

Heavy metals constitute a heterogeneous group of elements widely varied in their chemical properties and biological functions. Some of these are micronutrients necessary for plant growth, such as zinc, copper, manganese, nickel, and cobalt, while others like cadmium, lead, and mercury are known to cause serious, life-threatening health hazards (1). Amongst the heavy metals, lead has been used for centuries in many objects found in and around the home. Therefore, lead has been used to represent the heavy metal population in this project. Unlike organic contaminants, which are oxidized to carbon dioxide by microbial action, most metals do not undergo microbial or chemical degradation; instead, their total concentration in soils persists for a long time after their introduction. Because of their high degree of toxicity, lead along with other heavy metals like arsenic, cadmium, chromium, and mercury are considered systemic toxicants, as they can induce multiple organ damage even at low levels of exposure (1). Lead occurs naturally in soils, typically at concentrations that range from 10 to 50 mg/kg. Those lead concentrations are generally harmless, while lead concentrations of 400 ppm or more cause serious threats to human health, and the health hazards proportionally increase with higher lead concentrations **(Table 1)** (2).

According to the Environmental Protection Agency, it is estimated that there are almost half a million heavy metal-contaminated sites throughout the United States, and more than 217,000 of them are still in need of remediation. Several methods have already been used to clean up the environment from these kinds of contaminants, but most of them such as land filling, vitrification, chemical treatment, and electrokinetics are expensive costing up to $500 per ton of soil and need expensive maintenance such as transport, excavation, long term monitoring and recycling (2).

Rhizoremediation has the potential to be used as an effective and affordable technological solution that is

Table 1. The positive relationship between solid lead concentrations and toxicity.

Soil lead level (ppm)	Toxicity level (relative level of lead contamination)
Less than 150	None to very low
From 150 - 400	Low
From 400 - 1000	Medium
From 1000 - 2000	High
Greater than 2000	Very high

NOTE: Soil lead level of less than 150 part per million (ppm) generally does not cause any health hazards, but with lead levels above 400 ppm, health hazards start to proportionately increase with higher concentrations (2).

environmentally friendly and cost-effective. Rhizoremediation is a process that involves the degradation and detoxification of heavy metals and other pollutants by Plant Growth-Promoting Microbes (PGPM) in the rhizosphere layer of the soil (3). The rhizosphere is the soil zone surrounding the plant roots. Plants take up water and nutrients through the rhizosphere, and the microorganisms interact with exudates secreted by the roots (3, 4). The microbiome of the rhizosphere is the complex microbial ecosystem that nourishes the terrestrial biosphere, supporting estimates of 1,011 microbial cells per gram of root (4). Many of these microbes are tolerant of heavy metals and are termed Heavy Metal Tolerant PGPB (HMTPGPB). HMTPGPB alters metal bioavailability in the soil through various metal-microbial interactions such as bioaccumulation, bioleaching, and biosorption (4–6).

Although rhizoremediation is a well-known technique, not enough research exists in this field to enable its full utilization for heavy metal remediation. The microbiome of the soil *rhizobium* is extraordinarily complex, so there is a pressing need to scientifically design experiments for rhizoremediation by using specific plants and soil microbe combinations to detoxify heavy metals in soils in a controlled, simulated environment (7).

The goal of the project was to determine whether a much higher reduction in soil lead content can be observed when selective soil microbes are cultivated in the rhizosphere of *Brassica juncea, Oryza sativa,* or *Pisum sativum* in lead contaminated soil, than when no microbes are cultivated. Amongst several plants that were potentially suitable for rhizoremediation, *Brassica juncea* (Indian Mustard), *Oryza sativa* (rice), and *Pisum sativum* (peas) have proven the most promising. *Brassica juncea* has been proven to be a highly effective hyperaccumulator of heavy metals, as they can grow fast and attaining high biomass even under environmentally stressful conditions (7, 8). *Oryza sativa* is the most popular grain crop across the globe, and *Pisum sativum* is a popular leguminous plant consumed widely. The rationale to use both hyperaccumulators and non-hyperaccumulators was to determine if the rhizoremediation abilities varied between different plant species. If the crop species were successful in rhizoremediation, then our method would provide the opportunity to convert heavy metal contaminated nonarable lands to arable lands and increase crop production to reduce world hunger. *Pseudomonas* is one of the most common pathogens involved in dense biofilm formation. Microbial Extracellular Polymeric Substances (EPS) can bind significant amounts of heavy metals, thus inactivating them and preventing absorption of these heavy metals by the plants. In addition, many *Pseudomonas* species secrete a fluorescent yellow-green siderophore called pyoverdine, which chelates numerous heavy metals (9).

Rhizobia are a group of nitrogen-fixing bacteria that form symbiotic association with leguminous crop plants by forming nodules. These nodules are known to serve as metal buffer areas, which provide plants with an extra place to stock metals and reduce the risk of direct exposure of the plant to metals (9). *Burkholderia* strains can produce indoleacetic acid (IAA), a chemical that has the potential to solubilize inorganic phosphates that facilitate the uptake of the metals from soil (10). In addition, exopolysaccharides and siderophore production by *Burkholderia* strains have also been shown to promote heavy metal extraction from soil (10).

Based on the literature review and results obtained by data analysis from the National Center for Biotechnology Information (NCBI) that showed the bacterial composition of heavy metal-contaminated soil samples, we sought to test the hypothesis that if heavy metal tolerant soil microbes such as *Pseudomonas fluorescens, Burkholderia vietnamiensis,* and *Rhizobium leguminosarum* were cultivated in the rhizospheres of *Brassica juncea, Oryza sativa,* or *Pisum sativum* in lead contaminated soil, then a significant reduction in soil lead content could be observed (11). At the end of the procedure, we observed a remarkable decline in the soil lead content, with the highest decline in the triple microbe-plant combination with *Pseudomonas-Rhizobium-Burkholderia-Brassica juncea*.

RESULTS

Before beginning the rhizoremediation process, it was essential to determine the bacterial composition of the soil in lead-contaminated regions. We used publicly available data from NCBI to determine the composition of bacteria in the rhizosphere layer in several lead-contaminated and non-contaminated soil regions. We were able to get raw sequences of 16s rRNA from 96 soil samples. Then, using Qiime2, these raw sequences were demultiplexed and then denoised to obtain a taxonomic classification of the data. We then generated taxonomic bar plot from the results of Qiime2 analysis demonstrating the predominant microbial community in the contaminated vs non-contaminated rhizosphere **(Figure 1).**

Once the soil microbes were identified from the Qiime2 analysis, they were inoculated in the rhizosphere of the experimental plants in various combinations in lead-contaminated soil. We also included two control pots – one serving as negative control consisting of soil with lead and no plants or microbes and the other serving as a positive control that included soil with only plants from each group, but without any lead or microbes. The negative control pot was used to analyze soil lead content, whereas the positive control pots were used chlorophyll content analysis. Soil lead content was then measured at various stages of plant growth in both the experimental and negative control pots. Results of the pot experiment were analyzed in each group.

The effect of both the soil microbes and plants was significant (p = 0.02443), indicating that both soil microbes and plants have significant effect on lowering the soil lead level, and the results were not simply due to chance. Individually, results demonstrated significant reduction in soil lead content in these groups; *Pseudomonas-Burkholderia-*

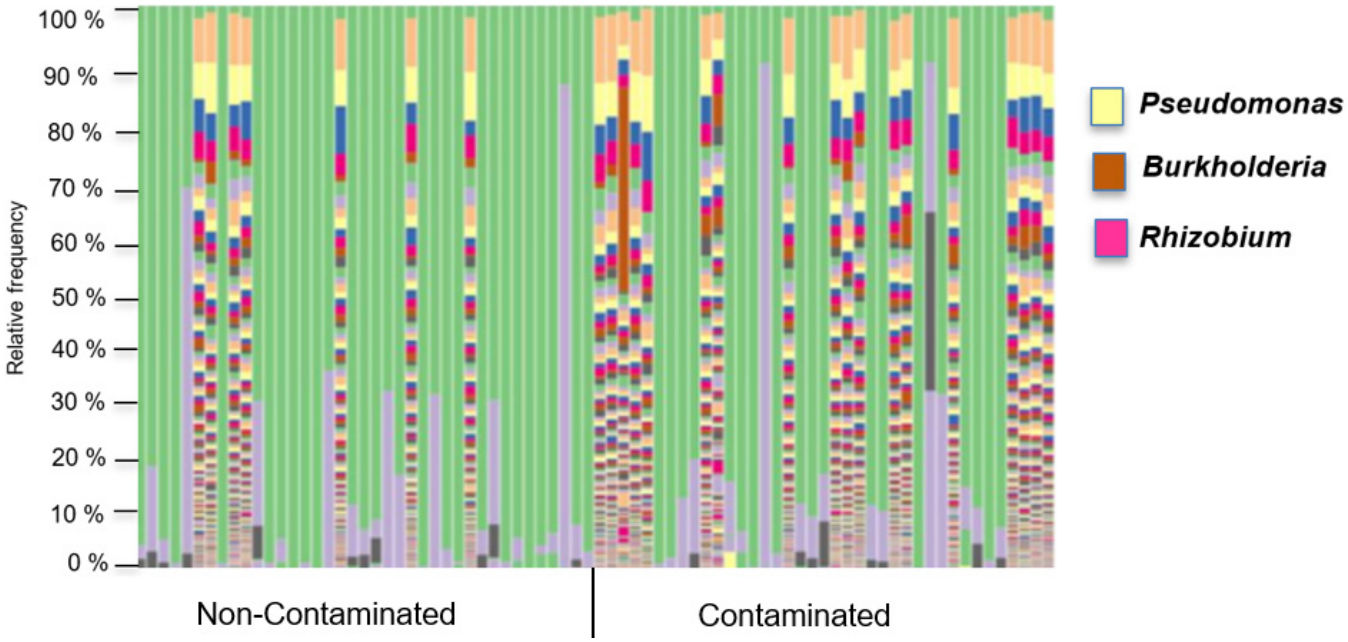

Figure 1. Bacterial composition of heavy metal-contaminated and non-contaminated soil samples. Taxonomic bar plot derived from 96 soil samples from NCBI dataset showing the relative frequency of bacterial genera (*Rhizobium*, *Pseudomonas*, and *Burkholderia*) present in contaminated vs. non-contaminated soil rhizosphere. These genera constituted over 43% of the microbial community in the contaminated rhizosphere. Green bars in the bar plot indicate no genera isolated. The other color codes indicate unidentified genera. Multiple boxes with the same color within each bar indicate the presence of multiple taxonomic groups at that particular level.

Rhizobium and Indian Mustard from 500 ppm to 50 ppm (p = 0.0245), *Pseudomonas-Burkholderia-Rhizobium* and rice from 500 ppm to 100 ppm (p = 0.0389), *Pseudomonas-Burkholderia-Rhizobium* and peas from 500 ppm to 100 ppm (p = 0.0375). The highest reduction in soil lead content, with a 90% reduction from 500 ppm to 50 ppm, was noted with *Pseudomonas-Burkholderia-Rhizobium* and Indian Mustard combination **(Table 2)**. There was also a notable decline in soil lead content in the following groups. We observed a 70% decline with *Pseudomonas-Brassica juncea* combination after four weeks, with a difference in lead content from 500 ppm to 150 ppm (p = 0.0572) **(Table 3)**. There was also a 65% decline in soil lead with *Burkholderia-Oryza sativa* combination, with a reduction from 500 ppm to 175 ppm (p = 0.0581) **(Table 4)**. Lastly, we observed a 60% decline with *Rhizobium-Pisum sativum* combination, with a reduction from 500 ppm to 200 ppm (p = 0.0598) **(Table 5)**.

Spectrophotometric analysis of the chlorophyll content of the dried leaves of all plant groups including the positive control with no lead in soil showed similar optical density (OD), indicating that the introduction of microbes and the lead decontamination in the soil did not negatively affect the plant

Table 2. Soil lead levels with *P. fluorescens* + *B. vietnamiensis* + *R. leguminosarum*.

	Control	Indian Mustard (*Brassica juncea*)	Rice (*Oryza sativa*)	Peas (*Pisum sativum*)
Week 0	500 ppm	425 ± 75 ppm	450 ± 50 ppm	475 ± 25 ppm
Week 2	500 ppm	200 ± 50 ppm	300 ± 25 ppm	300 ± 50 ppm
Week 3	500 ppm	100 ± 50 ppm	150 ± 75 ppm	200 ± 75 ppm
Week 4	500 ppm	**50 ± 25 ppm**	100 ± 50 ppm	100 ± 50 ppm

NOTE: The amount of lead (ppm) remaining in the soil at the end of each week was the least with *Pseudomonas fluorescens* + *Burkholderia vietnamiensis* + *Rhizobium leguminosarum* and Indian Mustard, compared to Rice or Peas, showing that the triple combination of microbes with Brassica juncea worked best in reducing the soil lead level. Bolded value indicates the least amount of lead, and the average values with corresponding standard deviations are listed in the table.

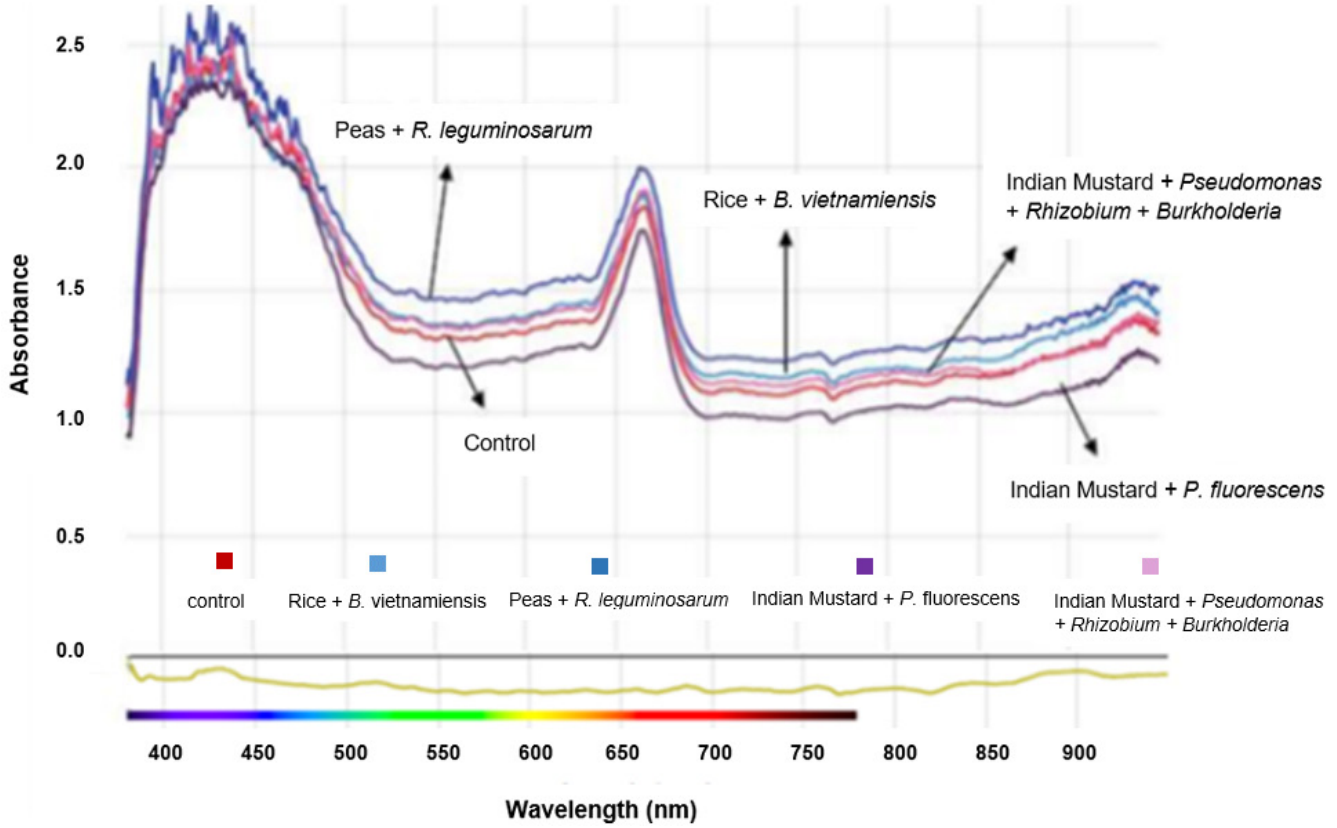

Figure 2. Absorbance values measured by optical density. The absorbance values (OD) of the chlorophyll content of all plants after 4 weeks of growth in soil contaminated with lead including positive control with Indian Mustard (with no lead in soil) was similar with no major variations.

health (**Figure 2).** Statistical analysis was not conducted for this part of the procedure.

DISCUSSION

To untangle the complexity of the rhizosphere microbiome, we obtained raw sequences of 16s rRNA from 96 soil samples from NCBI, which were then processed through Qiime2. We demonstrated that certain genera of bacteria (*Rhizobiales, Pseudomonas* and *Burkholderia* predominated in rhizospheres contaminated with heavy metal, constituting nearly 43% of the soil microbiome, showing a potential correlation to their ability to remediate heavy metals in the soil. *Pseudomonas fluorescens, Burkholderia vietnamiensis,* and *Rhizobium leguminosarum* were chosen as the experimental

Table 3. Soil lead levels with *Pseudomonas fluorescens.*

	Control	Indian Mustard (*Brassica juncea*)	Rice (*Oryza sativa*)	Peas (*Pisum sativum*)
Week 0	500 ppm	450 ± 50 ppm	425 ± 50 ppm	400 ± 75 ppm
Week 2	500 ppm	425 ± 25 ppm	450 ± 25 ppm	400 ± 25 ppm
Week 3	500 ppm	200 ± 75 ppm	400 ± 50 ppm	450 ± 25 ppm
Week 4	500 ppm	**150 ± 25 ppm**	250 ± 25 ppm	300 ± 50 ppm

NOTE: The amount of lead (ppm) remaining at the end of each week in the soil was the least with *Pseudomonas fluorescens* - Indian Mustard combination. Bolded value indicates the least amount of lead, and the average values with corresponding standard deviations are listed in the table.

Table 4. Soil lead levels with *Burkholderia vietnamiensis.*

	Control	Indian Mustard (*Brassica juncea*)	Rice (*Oryza sativa*)	Peas (*Pisum sativum*)
Week 0	500 ppm	450 ± 50 ppm	425 ± 75 ppm	450 ± 50 ppm
Week 2	500 ppm	400 ± 25 ppm	450 ± 25 ppm	300 ± 50 ppm
Week 3	500 ppm	300 ± 75 ppm	250 ± 50 ppm	250 ± 25 ppm
Week 4	500 ppm	200 ± 25 ppm	**175 ± 25 ppm**	200 ± 25 ppm

NOTE: The amount of lead (ppm) remaining in the soil at the end of each week was the least with *Burkholderia vietnamiensis* - rice combination. Bolded value indicates the least amount of lead, and the average values with corresponding standard deviations are listed in the table.

microbes for soil inoculation.

Among the individual microbe-plant combinations, the *Pseudomonas-Brassica juncea*, *Rhizobium-Pisum sativum,* and *Burkholderia-Oryza sativa* combination reduced the heavy metal contamination the most. After 4 weeks, we observed a 70% decline in the soil lead content in pots with *Pseudomonas-Brassica juncea*, a 65% decline with *Burkholderia-Oryza sativa,* and a 60% decline with *Rhizobium-Pisum sativum* combination. Other combinations showed a 50–60% reduction in soil lead content. The differences observed in the best performing pairs are likely due to symbiosis between the microorganisms and plants.

Among the triple microbe-plant combinations, soil lead content decreased from 500 ppm to 50 ppm in pots with *Pseudomonas-Rhizobium-Burkholderia-Brassica juncea*, a 90% decline in soil lead content, followed by *Pseudomonas-Rhizobium-Burkholderia-Oryza sativa* and *Pisum sativum*, where the soil lead content dropped from 500 ppm to 100 ppm — an 80% reduction in soil lead content. A slightly higher decline in soil lead content with *Brassica juncea* is likely because Indian Mustard is a hyperaccumulator. Spectrophotometric analysis of the chlorophyll content of the dried leaves of all plant groups including the positive control with no lead in the soil showed indicated that the lead decontamination in the soil did not negatively affect the plant health.

Amongst various plants, *Brassica juncea* outperformed all the other plants, as they can grow fast and attain high biomass even under environmentally stressful conditions. In addition, *Brassica* species have higher glutathione gene expression, which is the precursor of the heavy metal-binding peptides, resulting in heavy metal tolerance and thus facilitating detoxification of heavy metals (12).

In all soil microbe-plant combinations, the amount of lead in the soil decreased over time. Microbes used in combination were much more effective than when used individually with nearly 90% of lead extracted from the soil by week four. This can be explained by different pathways adapted by each of the microbes, which likely complement each other.

The results can be translated into developing a sustainable, ecofriendly, and cost-effective solution for the elimination of environmental contaminants from the soil. Amongst several

Table 5. Soil lead levels with *Rhizobium leguminosarum.*

	Control	Indian Mustard (*Brassica juncea*)	Rice (*Oryza sativa*)	Peas (*Pisum sativum*)
Week 0	500 ppm	475 ± 25 ppm	450 ± 50 ppm	425 ± 50 ppm
Week 2	500 ppm	400 ± 50 ppm	400 ± 25 ppm	400 ± 25 ppm
Week 3	500 ppm	300 ± 25 ppm	300 ± 50 ppm	250 ± 50 ppm
Week 4	500 ppm	225 ± 25 ppm	250 ± 75 ppm	**200 ± 25 ppm**

NOTE: The amount of lead (ppm) remaining in the soil at the end of each week was the least with *Rhizobium leguminosarum* - peas combination. Bolded value indicates the least amount of lead, and the average values with corresponding standard deviations are listed in the table.

methods of heavy metal remediation, rhizoremediation seems to be the most promising approach, as it is not only economical but also enables soil decontamination *in situ* without requiring additional steps to incinerate the plants. This process can be applied to any plants (including crop plants), not just to hyperaccumulators (several non-crop plants, which effectively accumulate heavy metals, but are otherwise not useful for consumption), and therefore can be utilized to not only clean the environment of toxic heavy metals but also to improve crop yield in the vast non-arable lands.

MATERIALS AND METHODS

Computational analyses

We obtained raw sequences of 16s rRNA from 96 soil samples that were heavy metal-contaminated and non-contaminated regions and geographically close to one another from NCBI (Taxonomy ID: 939928, 980194, 980190, 980128). Bacterial 16s rRNA genes were sequenced from different hypervariable regions (generally V4 or V3-V5). Raw sequences were truncated during the denoising process using DADA2. Finally, paired-end sequencing data was imported in Qiime2 to investigate the taxonomy of these strains and taxonomic bar plots were generated.

Preparing 500 ppm solution of lead using lead nitrate

The mass of lead nitrate required was corrected by multiplying the mass of lead with (Molecular weight of Pb $(NO_3)_2$ /Atomic weight of Pb^+) while weighing lead nitrate. To obtain 500 ppm of lead, 0.8 g of Pb $(NO_3)_2$ was dissolved in 1 L of distilled water.

Testing the lead content using the standard protocol listed in the Abotex lead testing kit

Commercially available Miracle-Gro potting soil was used for experimentation. Thirty-two oz clear plastic jars were used as pots for the plants and soil. One teaspoon of soil from the pot was taken and left to air dry overnight. The vials were half filled with soil and mixed with lead testing reagent. After 5 seconds, the color change in the test strip was compared with the color chart that was provided in the lead testing kit by the manufacturer. The waiting time was increased in 30-second increments for every 25 ppm tested (e.g., 2 min for 500 ppm, 4 min for 400 ppm, 6 min for 200 ppm, 7 min for 100 ppm, 8 min for 50 ppm, 10 minutes for 25 ppm).

Preparing bacterial inoculum

Pseudomonas fluorescens, Rhizobium leguminosarum, and *Burkholderia vietnamiensis* were grown in appropriate LB agar plates. After 48–72 hours incubation at room temperature (20°C), bacteria were collected from the culture plate using a standard inoculation loop, inoculated in 10 mL tryptic soy broth, and gently vortexed to make a homogeneous bacterial solution.

Potting experiment

We worked with three kinds of plants in this experiment: *Brassica juncea*, *Oryza sativa*, and *Pisum sativum*. Each plant was planted in four of each of the seventeen pots, including three pots treated with *Pseudomonas fluorescens*, three pots with *Rhizobium leguminosarum*, three pots with *Burkholderia vietnamiensis*, three pots with a combination of all three bacteria, and one pot in each plant group without bacteria or lead serving as a control for chlorophyll content analysis. In addition, we included a pot with just soil and lead without bacteria or plant to serve as a control for soil lead analysis. All pots were filled with commercially purchased 'Miracle-Gro' potting soil. Experimental pots were filled with 300 mL of lead nitrate. Using the Abotex lead testing kit, baseline lead content was measured to ensure it reflected 500 ppm. Three to five seeds of each plant were potted in each pot except the control pot for soil lead analysis. Seeds were watered with 250 mL distilled water each day. After 4–7 days (germination), 15 mL of each of the 3 bacterial inoculums and 5 mL combination of each of the three bacterial inoculums were poured around the seedling in the soil in each of the 9 experimental pots in every plant group. Soil lead content was measured in each pot including the control at the end of after two weeks, three weeks, and four weeks of experiment.

Analyzing the chlorophyll content of the leaves

Five dried leaves from each experimental and control group were obtained and placed in a plastic bag. Thirty mL of isopropyl alcohol was poured into each bag, and leaves were crushed gently to obtain green liquid extract, which was filtered using a coffee filter. Leaf extract was poured into plastic cuvettes. The spectrophotometer was calibrated using isopropyl alcohol and the optical density of each test group was analyzed to determine the chlorophyll a and b content, with the excitation wavelength of chlorophyll a at 614 nm and that of Chlorophyll b at 435 nm for all plant groups.

Statistical analysis

A two-way analysis of variance (ANOVA) test was conducted to examine the effects of both soil microbes and plants on soil lead level. Excel spreadsheet was used for this test. The independent variables in the analysis included three different microbes (*Rhizobium*, *Pseudomonas*, and *Burkholderia*) and three different plant groups (rice, peas, and Indian Mustard). Tukey's Honestly Significant Difference (HSD) post-hoc test was further used to determine if there was a significant difference in the results between the individual groups that were experimented on.

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