

Research article

Development of a Biofertilizer from Indigenous PGPR and Leonardite Residues for Sustainable Lettuce Cultivation and Soil Restoration

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Received: 24 June 2025, Revised: 8 October 2025, Accepted: 20 October 2025, Published: 30 January 2026

Abstract

Plant growth-promoting rhizobacteria (PGPR) are beneficial microbes that can enhance crop yield and soil fertility, particularly in degraded or mining-affected soils. In this study, we developed a biofertilizer using an indigenous PGPR strain, *Microbacterium maritypicum* R4-3, isolated from leonardite-associated rhizosphere at the Mae Moh lignite mine in Thailand. The strain showed strong phosphate solubilization (index = 3.81), siderophore production (1.43), and IAA synthesis (3.76 µg/mL). Its identity was confirmed by 16S rRNA sequencing and phylogenetic analysis as *M. maritypicum*. Lettuce (*Lactuca sativa* var. *crispa*) was used as a test crop. In laboratory experiments using Leonard's jar systems, R4-3 significantly increased plant biomass, yielding 18.58 g fresh weight (FW) and 1.97 g dry weight (DW) per plant, compared to 12.62 g FW and 1.36 g DW in uninoculated controls. In pot experiments with amended soils, the highest yield was obtained from 25% leonardite plus R4-3, reaching 48.68 g FW and 2.92 g DW, outperforming both controls (1.94 g FW, 0.18 g DW) and two commercial biofertilizers. Soil fertility improved significantly ($p < 0.05$), with higher total nitrogen (0.41%), exchangeable potassium (448 ppm), and organic matter (7.9%), along with pH stabilization. These effects are attributed to nutrient mobilization and enhanced microbial activity by R4-3. The findings demonstrate the dual role of *M. maritypicum* R4-3 as both a plant growth enhancer and a soil-restoring agent, providing a sustainable, low-cost strategy for converting mining by-products into effective biofertilizer inputs for organic farming and land rehabilitation.

Keywords: PGPR; leonardite; rhizosphere; *Microbacterium*; biofertilizer; soil restoration; lettuce; organic amendment

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<https://doi.org/10.55003/cast.2026.268134>

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1. Introduction

Lignite mining produces large amounts of waste by-products, particularly leonardite and red clay. Leonardite, a naturally oxidized form of lignite, is rich in humic substances that can improve soil structure and nutrient availability (Savarese et al., 2022). In contrast, red clay is a mineral-rich by-product with minimal organic matter and is often treated as inert waste. Despite their contrasting compositions and ready availability, both materials have been largely overlooked for agricultural applications. Moreover, the microbial communities associated with these substrates, particularly those possessing plant-beneficial traits, remain insufficiently characterized. This gap in knowledge limits their practical integration into soil restoration and sustainable crop production. Their reuse as soil amendments may offer a practical solution for reducing mining waste and supporting circular economy practices.

Plant growth-promoting rhizobacteria (PGPR) are beneficial soil microbes that enhance plant growth through various mechanisms, such as nitrogen fixation, phosphate solubilization, potassium mobilization, and indole-3-acetic acid (IAA) production (Grover et al., 2021; Zuluaga et al., 2024). Beyond facilitating nutrient acquisition, PGPR also play roles in alleviating abiotic stress and promoting overall soil health (Malik et al., 2024). Due to these diverse functions, PGPR have been extensively investigated as core agents in biofertilizers, which serve as eco-friendly alternatives to chemical fertilizers in sustainable agriculture and land rehabilitation.

Several *Microbacterium* species have shown potential as bioinoculants under diverse agricultural and environmental conditions. For example, strain SRS2 improved *Arabidopsis* and tomato growth under salt stress (Tuong et al., 2024), while *M. azadirachtae* CNUC13 enhanced maize performance in saline soils (Luo et al., 2024). These findings underscore *Microbacterium*'s potential as stress-tolerant PGPR. Nevertheless, their application in substrates derived from lignite mining, such as leonardite or red clay, remains largely unexplored. Harnessing such indigenous microbes could expand the development of localized, low-cost biofertilizers for degraded environments.

Combining PGPR with organic amendments such as leonardite may further enhance microbial efficacy by improving habitat conditions and nutrient supply. The humic substances present in leonardite can promote root colonization, stimulate microbial metabolism, and increase the expression of growth-promoting traits. Previous studies have reported that humic acids can induce auxin production and improve nutrient solubilization in PGPR (Pantoja-Guerra et al., 2024; Tsavkelova et al., 2024). However, the potential synergistic effects between PGPR and leonardite in mine-derived substrates have not been systematically examined.

This study aimed to investigate the potential of indigenous PGPR isolated from leonardite and red clay in the Mae Moh lignite mine in northern Thailand. The objectives were to (1) isolate and identify PGPR strains from rhizosphere soils associated with mining residues, (2) characterize their plant growth-promoting traits, (3) assess their effects on lettuce (*Lactuca sativa* var. *crispa*) growth under controlled and pot conditions, and (4) compare their performance with that of commercial biofertilizers. Lettuce was selected as a model crop due to its fast growth and sensitivity to soil nutrients, making it a suitable indicator of microbial biofertilizer effectiveness. The study also sought to explore whether co-application of PGPR and leonardite could serve as a practical strategy for developing sustainable biofertilizers while promoting soil restoration in mining-affected landscapes.

2. Materials and Methods

2.1 Soil sampling and bacterial isolation

Rhizosphere soil samples were collected from plant roots growing in leonardite and red clay deposits at the Mae Moh lignite mine, Lampang, Thailand (18.3304, 99.7103) (Figure 1). Samples were taken from visibly healthy plants growing naturally on the substrate surfaces. Host plants and corresponding 12 isolates included: *Sesbania javanica* (M1-1), *Macroptilium lathyroides* (R1-1), *Gomphrena celosioides* (R2-1), *Ageratum conyzoides* (R3-1), *Paederia foetida* (R4-1, R4-3), *Mimosa pudica* (R5-1, R5-2), and *Eleusine indica* (R6-1) from leonardite, and *M. lathyroides* (R7-1, R7-2), *G. celosioides* (R7-3) from red clay. One gram of each soil sample was suspended in 9 mL of sterile 0.85% NaCl solution and shaken for 30 min. Serial dilutions were spread on nitrogen-free LG agar to selectively isolate free-living diazotrophs, considered one of the PGP traits (Piromyou et al., 2011). Plates were incubated at 28-30°C for 1-3 days. Morphologically distinct colonies were selected and maintained for further analysis. Leonardite and red clay were also collected from the sampling sites for further pot experiment.

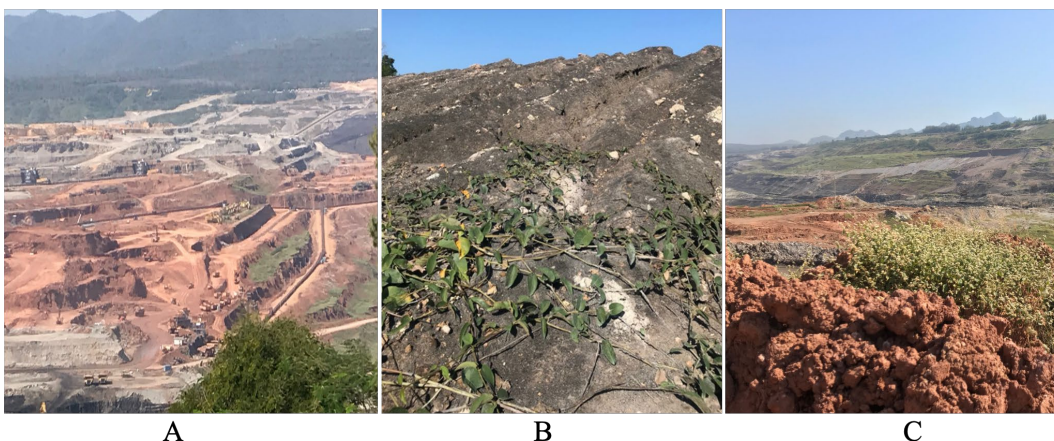


Figure 1. Plant growth observed at the lignite mine sampling sites (A) where rhizosphere soil was collected in leonardite deposits (B) and red soil (C) pile.

After initial isolation, 12 morphologically distinct colonies were selected based on differences in colony size, shape, margin, elevation, color, and texture. Genetic diversity among these isolates was further confirmed by BOX-PCR fingerprinting, which demonstrated distinct banding patterns for each isolate, indicating 12 genetically unique strains. Subsequently, all 12 isolates were screened for key plant growth-promoting (PGP) traits, including phosphate solubilization, siderophore production, and indole-3-acetic acid (IAA) synthesis. Seven isolates exhibiting at least one or more positive PGP activities were selected for further evaluation in pot experiments and for 16S rRNA gene sequencing.

2.2 Molecular characterization of PGPR isolates

Genomic DNA was extracted from selected bacterial isolates using the DNA Mini Kit (Qiagen). To eliminate duplicate isolates, genomic DNA fingerprints of each bacterial isolate were analyzed using BOX-PCR with the BOX-A1R primer (5'-CTA CGG CAA GGC GAC GCT GAC G-3'). PCR reactions were carried out in 25 µL volumes containing 10 pmol of primer, 2.5 mM of each dNTP, 25 mM MgCl₂, 0.5 U Taq DNA polymerase, and 1× Taq polymerase buffer. The amplification was performed in a thermal cycler (Biostep, Argus X1 version 7, USA) under the following cycling conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 8 min, and extension at 65°C for 16 min. The PCR products were analyzed by gel electrophoresis on 2% agarose gel at 100 V for 60 min. DNA banding patterns were visualized using a gel documentation system (UVITEC Fire Reader, UK). Isolates showing unique banding profiles were selected for further analysis. Cluster analysis and dendrogram construction based on BOX-PCR band patterns were performed using UVbandmap software (Uvitec Cambridge, UK) to assess genetic relationships among the isolates.

Genomic DNA from genetically distinct isolates was used for 16S rRNA gene amplification with universal primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rP2 (5'-ACG GCT ACC TTG TTA CGA CTT-3') (IDT, Singapore). PCR products were confirmed by agarose gel electrophoresis and purified using the GeneJET Gel Extraction Kit (Thermo Scientific, USA) following the manufacturer's protocol. Purified PCR products were subjected to Sanger sequencing (First BASE, Malaysia). The resulting sequences were identified through comparison with the NCBI GenBank database using the BLASTn algorithm.

The phylogenetic trees were constructed in MEGA12 software using the Tamura–Nei model and the Maximum Likelihood method with 1,000 bootstrap replicates. The nucleotide sequences obtained were deposited in GenBank database under accession numbers PV569535 to PV569541.

2.3 Screening of plant growth-promoting traits

Bacterial isolates were screened for plant growth-promoting (PGP) traits including indole-3-acetic acid (IAA) production, phosphate solubilization, and siderophore production.

IAA production was measured by culturing isolates in LG broth supplemented with 30.5 mg/L tryptophan at 30°C and agitation at 150 rpm for 5 days. Culture supernatants were mixed with Salkowski reagent, and absorbance was measured at 530 nm using a cuvette and a UV–Vis spectrophotometer. IAA concentrations were calculated using a standard curve prepared from pure IAA (Sigma-Aldrich) (Costacurta et al., 1998).

Phosphate solubilization was assessed on modified Pikovskaya's agar (Piromyou et al., 2011). Isolates were spot-inoculated and incubated at 30°C for 5 days. The solubilization index was calculated as the ratio of the halo diameter to the colony diameter after 24 h of incubation.

Siderophore production was evaluated on Chrome Azurol S (CAS) blue agar containing HDTMA. Plates were incubated at 30°C for 5 days. Siderophore activity was quantified based on the diameter of the yellow–orange halo surrounding each colony (Schwyn & Neilands, 1987).

2.4 Laboratory experiment

Red oak lettuce (*Lactuca sativa* var. *crispa*) seeds were surface sterilized by immersion in 1.5% sodium hypochlorite for 10 min, followed by two washes with sterile distilled water. Seeds were incubated in the dark at 20°C for 5 days until radicle emergence. Germinated seedlings were transferred into modified Leonard's jar systems, with the upper compartment containing sterilized vermiculite (4-8 mm, Agra-Vermiculite) and the lower compartment filled with nitrogen-free plant nutrient solution (Somasegaran & Hoben, 1994). Nutrient uptake occurred via capillary transport through cotton wick connections.

Seven PGPR isolates were cultured in LG broth and adjusted to 10^7 CFU/mL using sterile saline. Each seedling received 1 mL of bacterial suspension at transplanting; control plants received 1 mL of sterile saline. All treatments, including the uninoculated control, were arranged with ten replicates. Plants were grown under controlled conditions with a 10 h dark/14 h light cycle at 25-30°C. Regular monitoring and refilling of the solution reservoir maintained proper water content and ensured continuous nutrient supply for plant development. After 30 days, fresh and dry weights were measured. Isolate R4-3, which produced the highest dry biomass, was selected for further testing in pot experiments.

2.5 Pot experiment

2.5.1 Inoculum and substrate preparation

The R4-3 inoculum was prepared following the same protocol as in the laboratory experiment. Two commercial PGPR products, PGPR-2® and SB®, were applied at 1 g/plant according to manufacturer recommendations. The PGPR-2® contained *Azospirillum* spp. (1.0×10^6 cfu/g) and *Burkholderia* spp. (1.0×10^6 cfu/g), while SB® contained *Azospirillum brasilense* (1.0×10^6 cfu/g), *Azotobacter vinelandii* (1.0×10^6 cfu/g), and *Beijerinckia mobilis* (1.0×10^6 cfu/g). The base soil consisted of a 1:1 (v/v) mixture of loam soil and rice husk charcoal. Five substrate types were prepared by blending the base soil with leonardite or red clay at 25% and 50% (v/v): (1) base soil alone, (2) 25% leonardite + 75% soil, (3) 50% leonardite + 50% soil, (4) 25% red clay + 75% soil, and (5) 50% red clay + 50% soil. Each pot contained approximately 1.5 L of substrate. These amendment levels (25% and 50% v/v) were chosen to reflect practical field application and to help reduce waste accumulation at the mining site.

2.5.2 Planting and experimental design

Ten-day-old red oak lettuce seedlings, previously germinated in sterilized peatmoss trays, were transplanted into plastic pots (15 cm diameter × 11 cm height). The experiment was arranged as a two-factor factorial in a completely randomized design (CRD), with five substrate types and four PGPR treatments (Control, R4-3, PGPR-2®, and SB®), resulting in 20 treatment combinations. Each combination was replicated five times (n = 100 pots).

Bacterial inoculants were applied at transplanting: 1 mL of R4-3 suspension (10^7 CFU/mL) or 1 g of commercial product per plant. Additional applications were given on days 10 and 20. Pots were arranged in randomized rows in a greenhouse for 30 days with approximately 12 h of natural sunlight per day and irrigated once daily. Fresh and dry biomass were recorded 30 days after transplanting. Dry weights were determined after oven-drying at 70°C for 24 h.

2.6 Soil physicochemical analysis

Soil samples were collected from three substrate types (Soil, L25, and R25) at three sampling stages: before planting, after planting (uninoculated), and after planting with PGPR strain R4-3 treatment. For each treatment, three individual rhizosphere soil samples were randomly collected from three pots, representing three independent replicates ($n = 3$) per treatment. A total of 27 soil samples were analyzed. The collected soils were air-dried and sieved through a 2 mm mesh prior to analysis. Total nitrogen (N) was determined using the Kjeldahl method (Kjeltec 8100, FOSS). Exchangeable potassium (K) was extracted with 1 N ammonium acetate (NH_4OAc). Available phosphorus (P) was analyzed by the Bray II method using spectrophotometry, and organic matter (OM) was determined following the Walkley–Black procedure. Soil pH and electrical conductivity (EC) were measured in a 1:5 soil-to-water suspension using a digital pH/EC meter. All analyses were conducted at the Center for Scientific and Technological Equipment, Suranaree University of Technology, following standard analytical protocols.

2.7 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics (Version 26). For the Leonard's jar assay, fresh and dry biomass data were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) for mean separation at a significance level of $p < 0.05$. For the pot experiment, a two-way ANOVA was conducted under a completely randomized design (CRD), with PGPR treatment and substrate type as fixed factors. Interaction effects were also evaluated, and significant differences were further examined using DMRT.

3. Results and Discussion

3.1 Molecular identification and PGP traits of isolates

A total of 12 bacterial isolates were obtained from rhizosphere soils associated with plants growing on leonardite and red clay deposits. Morphologically distinct colonies were observed among the isolates. BOX-PCR fingerprinting confirmed their genetic uniqueness, revealing considerable strain-level diversity among culturable rhizobacteria in the mining residues.

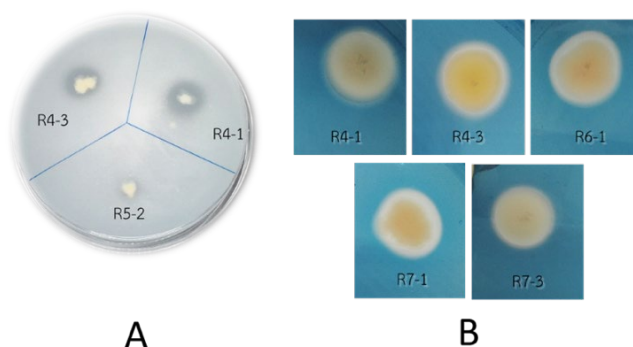
Seven isolates exhibited at least one plant growth-promoting (PGP) trait, including phosphate solubilization, siderophore production, and IAA synthesis (Table 1, Figure 2). The 16S rRNA gene analysis identified the dominant isolates as *Microbacterium maritopicum* (R4-3), *Kosakonia oryzendophytica* (R4-1), *Enterobacter cloacae*, *Rhizobium nepotum*, and *Pseudomonas mendocina*.

Among the tested isolates, R4-3 showed the highest performance across three PGP traits, including phosphate solubilization, siderophore production, and IAA synthesis. These results highlight its multifunctional capacity under controlled *in vitro* conditions. Several studies have reported PGPR strains with one or more potent traits under specific conditions. For instance, chrysanthemum rhizobacteria exhibited strong IAA production and secretion of hydrolytic enzymes, enabling adaptation under osmotic stress (Chowdhury et al., 2024). Similarly, *Microbispora* sp. SCL1-1 strain from Thai soils could produce siderophores, IAA, and solubilize phosphate (Dejtisakdi et al., 2024). However, strain R4-3 demonstrates a broader spectrum of PGP activities under nutrient-limited and mining-amended conditions, supporting its multifunctional potential in stressed environments.

Table 1. Molecular characterization of PGPR isolates using 16S rRNA gene amplification and plant growth-promoting traits of bacterial isolates

Isolate	Identified Species	Phosphate Index	Siderophore Index	IAA ($\mu\text{g/mL}$)
M1-1	99.52% <i>Rhizobium nepotum</i>	-	-	0.13 $\pm$ 0.04 ^c
R4-1	99.64% <i>Kosakonia oryzendophytica</i>	3.28 $\pm$ 0.03 ^b	1.17 $\pm$ 0.04 ^b	0.57 $\pm$ 0.05 ^b
R4-3	99.40% <i>Microbacterium maritypicum</i>	3.81 $\pm$ 0.03 ^a	1.43 $\pm$ 0.03 ^a	3.76 $\pm$ 0.03 ^a
R5-2	99.70% <i>Enterobacter cloacae</i> subsp. <i>dissolvens</i>	3.17 $\pm$ 0.06 ^b	-	0.05 $\pm$ 0.03 ^c
R6-1	99.10% <i>Pseudomonas mendocina</i>	-	1.27 $\pm$ 0.02 ^b	0.01 $\pm$ 0.02 ^c
R7-1	99.12% <i>Pseudomonas mendocina</i>	-	1.36 $\pm$ 0.04 ^b	0.01 $\pm$ 0.09 ^c
R7-3	99.68% <i>Enterobacter cloacae</i> subsp. <i>dissolvens</i>	-	1.22 $\pm$ 0.05 ^b	0.3 $\pm$ 0.06 ^{bc}

Note: Different lowercase letters within the same column indicate statistically significant differences among treatments at $p < 0.05$ according to DMRT.

**Figure 2.** Example of phosphate solubilization (A) and siderophore production (B) capabilities of PGPR

Phylogenetic analysis of the 16S rRNA gene confirmed that isolate R4-3 was closely related to *M. maritypicum* DSM 20638, with 100% bootstrap support (Figure 3). This strong genetic affiliation, combined with its distinctive PGP traits, identified R4-3 as a promising candidate for bioinoculant development. Isolate R4-3 was obtained from the rhizosphere of *P. foetida*, a medicinal liana known for its rich microbial community. Previous studies have reported diverse endophytic and rhizospheric bacteria from *P. foetida* with antimicrobial, improve crop performance and disease resistance (Patil et al., 2024), supporting the potential of R4-3 as a robust bioinoculant. The capacity of *Microbacterium* species to function as PGPR under stressful environmental conditions was recognized (Mandal et al., 2024; Yuan et al., 2024). Phylogenetic analysis based on 16S rRNA gene sequences confirmed the identity of the isolated strains. Notably, isolate R4-3 clustered tightly with *M. maritypicum* DSM 20638 with 100% bootstrap support, validating its classification. Similarly, isolates R4-1, R5-2, R7-3, R6-1, R7-1, and M1-1 grouped with their respective type strains (*Kosakonia*, *Enterobacter*, *Ectopseudomonas*, and *Rhizobium*) with high confidence. The inclusion of multiple reference strains and appropriate outgroups (e.g., *Bacillus subtilis*, *Streptomyces coelicolor*) further ensured robust phylogenetic resolution.

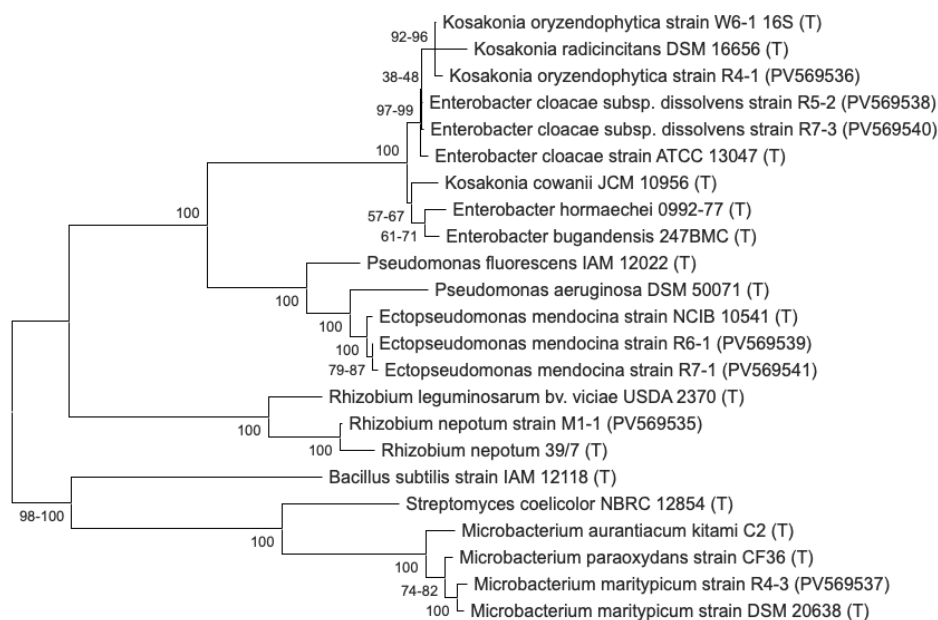


Figure 3. Phylogenetic tree based on 16S rRNA gene sequences showing relationships among PGPR isolates and type strains. The tree was constructed using the Maximum Likelihood method (Tamura–Nei model), with bootstrap values ($\geq 70\%$) from 1,000 replicates shown at nodes. Isolates from this study are marked by strain codes (e.g., R4-3, R4-1), while type strains are indicated with a superscript "T" and their GenBank accession numbers in parentheses.

3.2 Growth promotion in Leonard's jar assay

Application of PGPR isolates significantly enhanced red oak lettuce (*L. sativa* var. *crispa*) growth under nitrogen-free conditions in Leonard's jar assays ($p < 0.001$). Among all treatments, isolate R4-3 yielded the highest biomass, with fresh and dry weights of 18.58 ± 0.59 g/plant and 1.97 ± 0.07 g/plant, respectively. These values were significantly greater than those of the uninoculated control (12.62 ± 0.68 g and 1.36 ± 0.09 g), and higher than all other isolates tested (Figure 4).

R4-1 also promoted biomass accumulation, though to a lesser extent than R4-3, whereas other isolates such as R5-2, R6-1, and R7-1 yielded intermediate values. These results highlight the superior growth-promoting ability of R4-3, consistent with its strong PGP traits observed *in vitro*. These findings are consistent with previous reports demonstrating PGPR-mediated growth enhancement under nitrogen-limited conditions (Sun et al., 2024). Although the Leonard's jar system provides an artificial growth environment, it justified its selection for further evaluation in pot experiments involving more complex soil systems.

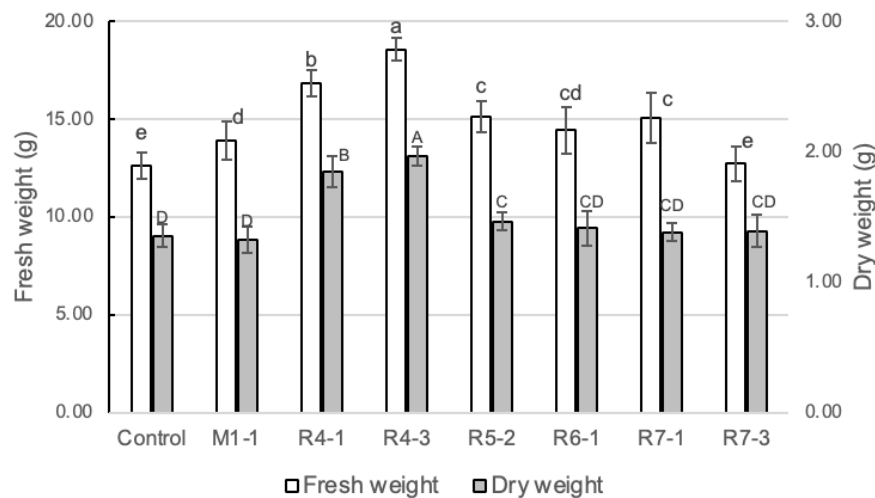


Figure 4. Fresh and dry biomass of red oak lettuce (*Lactuca sativa* var. *crispa*) grown under Leonard's jar assay with different PGPR isolates. Bars represent mean±standard error (n = 10). Different lowercase letters above fresh weight bars and uppercase letters above dry weight bars indicate significant differences among treatments at $p < 0.05$ according to DMRT.

3.3 Growth promotion in pot experiments

The two-way ANOVA confirmed that both substrate type ($F = 46.93$ for FW; 70.92 for DW, $p < 0.001$) and PGPR strain ($F = 145.70$ for FW; 255.70 for DW, $p < 0.001$) had highly significant effects on lettuce growth, with a strong interaction between the two factors ($F = 10.81$ for FW; 12.66 for DW, $p < 0.001$). These findings suggest that lettuce growth responses varied with substrate composition, and that the efficiency of PGPR inoculation depended strongly on the soil environment. Post-hoc comparison using DMRT confirmed that R4-3 treatment in 25% leonardite-amended soil (L25-R4-3) produced the highest fresh and dry biomass, significantly greater than all other treatment combinations ($p < 0.05$). Distinct statistical groupings further supported the superior performance of R4-3 across most substrates, especially in leonardite-amended conditions (Figures 5 and 6). This demonstrates that R4-3 consistently outperformed both commercial biofertilizers (PGPR-2® and SB®), particularly under mining-amended soil conditions. Its effectiveness was most evident at the 25% amendment level, while higher amendment rates (50%) led to substantial growth reductions, likely due to potential nutrient imbalances or substrate-induced phytotoxicity.

The present findings are consistent with previous reports showing that several *Microbacterium* species can enhance plant growth under stressful environments such as salinity and nutrient limitation (Alishahi et al., 2020; Luo et al., 2024; Tuong et al., 2024). These findings reinforce that strain R4-3 possesses ecological versatility typical of the genus, which may explain its strong performance in mining-affected soils. These results suggest that ecological matching between PGPR origin and soil environment plays a crucial role in determining biofertilizer efficacy. Consistent with this interpretation, previous

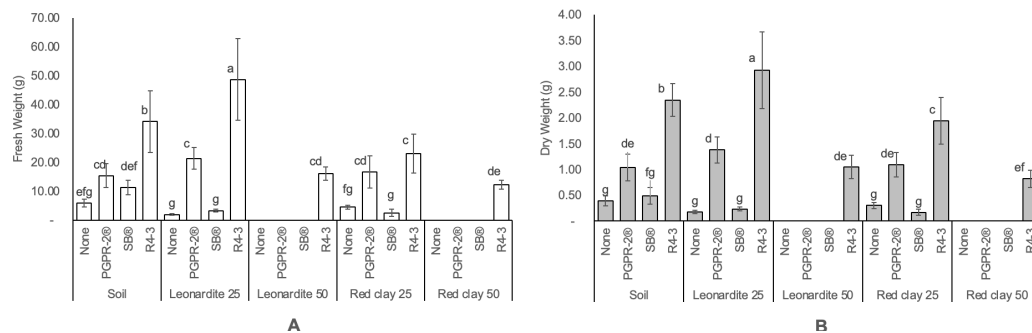


Figure 5. Fresh (A) and dry (B) biomass of red oak lettuce (*Lactuca sativa* var. *crispa*) under different PGPR treatments and substrate amendments in pot experiments. Bars represent mean±standard error (n = 5). Different lowercase letters above fresh weight bars and uppercase letters above dry weight bars indicate statistically significant differences among treatments according to DMRT (p<0.05). F-test results from two-way ANOVA confirmed significant main effects of substrate type, PGPR strain, and their interaction (p<0.001) on both fresh and dry biomass.

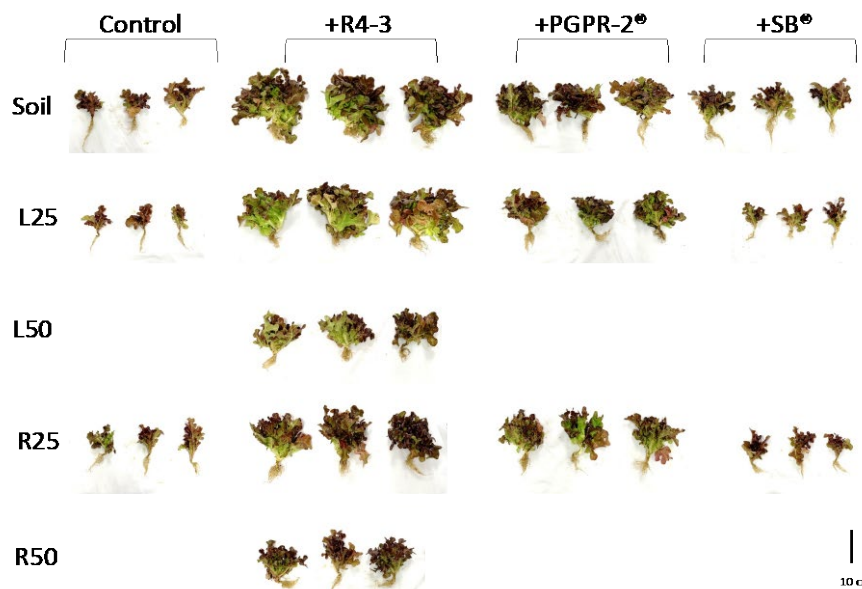


Figure 6. Growth performance of red oak lettuce under different PGPR treatments and soil substrate conditions in the pot experiment. Treatments included control (uninoculated), R4-3 (*Microbacterium maritropicum*), and two commercial biofertilizers (PGPR-2® and SB®), applied to five substrate types: unamended soil (soil), 25% and 50% leonardite-amended soil (L25, L50), and 25% and 50% red clay-amended soil (R25, R50). Representative plants were photographed 30 days after transplanting. The absence of plants in some treatments indicates plant mortality that occurred shortly after transplanting under certain substrate conditions. Scale bar = 10 cm.

studies showed that moderate concentrations of leonardite promoted plant growth in lettuce (*Lactuca sativa* L.), where root and foliar applications at 0.20-0.60 mL L⁻¹ and 5.00-10.00 mL L⁻¹ enhanced biomass, while higher rates (0.80 mL L⁻¹ root and 12.5 mL L⁻¹ foliar) suppressed growth and photosynthesis (Atero-Calvo et al., 2023). Similar patterns were observed in other crops: melon (*Cucumis melo* L.) seedlings showed marked inhibition when grown in 100% leonardite or a 50% leonardite–perlite mix (Kolay, 2025), and *Coix lacryma-jobi* L. exhibited reduced growth in high-proportion red clay substrates due to poor aeration and high bulk density (Liu et al., 2024). In *Amorphophallus muelleri*, mixing red clay with humus and sand alleviated this suppression (Xu et al., 2024). Together, these findings confirm that moderate leonardite or red clay amendments favor both R4-3 activity and lettuce growth, while excessive levels may inhibit plant performance.

The superior performance of R4-3 in non-sterile pot experiments indicates that its growth-promoting activity remains effective even in the presence of native soil microbiota. Similar findings were reported in other PGPR consortia that enhanced growth and disease resistance of *Arabidopsis* and wheat under both sterile and non-sterile soils (Hashmi et al., 2019; Samain et al., 2023), although efficacy was slightly reduced due to competition with native microbiota. This finding suggests that R4-3 is ecologically compatible with natural microbial communities, strengthening its field applicability.

3.4 Soil fertility improvements from PGPR–Leonardite interaction

Inoculation with R4-3, particularly in combination with 25% leonardite, markedly improved soil chemical properties compared with uninoculated controls. Total nitrogen, exchangeable potassium, and organic matter increased significantly, while available phosphorus remained relatively stable. Leonardite amendments lowered soil pH and increased EC, but R4-3 contributed to pH buffering, especially in red clay treatments. These results show a clear synergistic effect between R4-3 and leonardite in improving both plant productivity and soil nutrient status. This synergistic interaction may be attributed to improved microbial survival and metabolic activity in humic-rich environments, which enhance root development and nutrient mobilization. Co-application of PGPR with humic substances has been shown to increase auxin activity and root biomass (Savarese et al., 2022; Mandal et al., 2024; Pantoja-Guerra et al., 2024), and to stimulate the expression of growth-promoting genes involved in auxin synthesis and nutrient release (Bhutani et al., 2018; Grover et al., 2021).

Comparable effects were reported for other *Microbacterium* species. In restoration ecosystems, *M. invictum* isolated from *R. pseudoacacia* rhizosphere improved both plant growth and soil nutrient availability (Liu et al., 2022). The VOC-producing *M. aurantiacum* stimulated tobacco growth via hormonal pathways including auxin, salicylic acid, and gibberellin regulation (Gao et al., 2022). Compared to these habitat-specific isolates, R4-3 demonstrated stable performance under both nitrogen-deficient laboratory conditions and mining waste-amended substrates, indicating its broad adaptability and functional consistency across multiple stress factors. These characteristics emphasize the potential of R4-3 as a robust bioinoculant for plant growth promotion and soil rehabilitation in degraded post-mining environments.

The synergistic effect observed between R4-3 and leonardite may be attributed to humic substance–microbe interactions that enhance both microbial activity and plant growth. Humic substances present in leonardite can serve as carbon sources and signaling molecules, stimulating microbial metabolism, biofilm formation, and root colonization efficiency (Atero-Calvo et al., 2023; Piccolo & Drosos, 2025). In turn, PGPR activities such

as phosphate solubilization, IAA production, and siderophore secretion may be upregulated, leading to improved nutrient availability and hormonal balance within plants. Moreover, humic substances may directly enhance plant physiology by modulating auxin-like activity, promoting root elongation, and facilitating greater nutrient uptake.

3.5 Soil physicochemical changes following PGPR application

Soil chemical properties were evaluated before and after cultivation to assess the effects of PGPR inoculation and substrate amendments (Figure 7). Inoculation with R4-3 significantly increased total nitrogen, exchangeable potassium, and organic matter compared with controls. Two-way ANOVA confirmed significant effects and interactions for most parameters (Total N, K, OM, and pH; $p < 0.05$), except EC ($p \geq 0.05$). These improvements indicate that R4-3 effectively mobilized soil nutrients and stimulated microbial activity in the rhizosphere.

In particular, exchangeable potassium rose sharply in R4-3-treated soils, especially in L25+R4-3 (449 ppm) and R25+R4-3 (426 ppm), compared with uninoculated controls. This finding aligns with previous reports showing that potassium-solubilizing PGPR release organic acids that lower soil pH and dissolve K-bearing minerals, thereby converting non-exchangeable K into plant-available forms. For instance, inoculation with *Enterobacter cloacae* Rhizo_33 significantly increased soil available K by promoting mineral dissolution and releasing exchangeable forms (Khani et al., 2019). Similarly, PGPR may enhance K availability through several mechanisms, including organic acid production, chelation of metal ions, and biofilm formation that accelerates mineral weathering (Olaniyan et al., 2022). Together, these mechanisms help maintain higher soil K levels even under intensive plant uptake, explaining the high K concentrations observed in R4-3-treated soils.

Interestingly, available phosphorus levels remained relatively stable across treatments. This likely reflected simultaneous plant uptake and ongoing microbial solubilization, which maintained phosphorus availability without substantial accumulation. The stability of P despite increased biomass production suggests a potential role of R4-3 in maintaining P availability during crop uptake cycles, potentially through organic acid secretion or siderophore-mediated mechanisms. Comparable trends have been reported in tomato (*Solanum lycopersicum* L.), where inoculation with multiple PGPR strains significantly increased soil P availability due to phosphate-solubilizing activity (Rehan et al., 2023). PGPR are known to facilitate P cycling through mechanisms similar to those of K, including organic acid secretion, rhizosphere acidification, and mineral dissolution, as well as enzymatic processes (phosphatases, phytases, C-P lyases) regulated by genes such as *gcd* and *pqq* (Zeng et al., 2022).

Leonardite-amended substrates exhibited lower pH (6.06-6.68) and higher electrical conductivity (2.9-3.3 dS/m) than unamended soils. These conditions may enhance microbial activity and nutrient availability but can also impose osmotic stress at higher amendment rates, as reflected in the suppressed plant growth observed in 50% leonardite and red clay treatments.

In the L25 treatment, R4-3 increased total nitrogen by 10%, exchangeable K by 69%, and organic matter by 35% compared with the uninoculated control, indicating efficient nutrient mobilization and improved soil quality. Taken together, these results show that R4-3 inoculation, especially when combined with 25% leonardite, significantly improved soil fertility through increased nitrogen and potassium availability, enrichment of

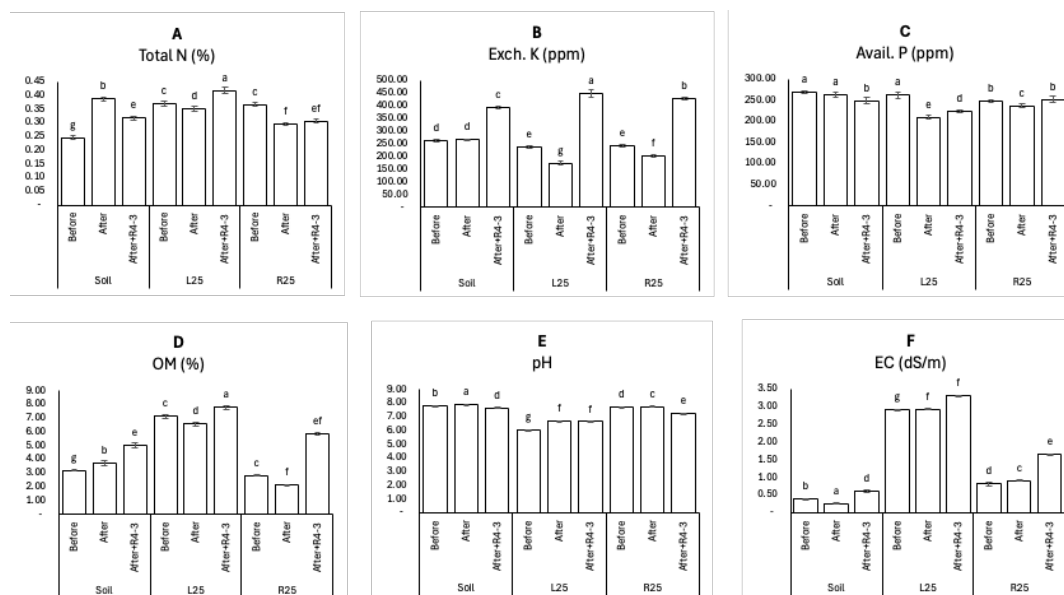


Figure 7. Soil physicochemical properties before planting, after planting (uninoculated), and after planting with PGPR strain R4-3 in different substrates (Soil, L25, and R25). Parameters include total nitrogen (A), exchangeable potassium (B), available phosphorus (C), organic matter (D), soil pH (E), and electrical conductivity (F). Bars represent mean $\pm$ standard error (n = 3). Different lowercase letters above bars indicate statistically significant differences among treatments within each parameter according to DMRT ($p < 0.05$). “Before” = soil before cultivation; “After” = soil sampled after cultivation without PGPR inoculation; “After+R4-3” = soil sampled after cultivation with R4-3 inoculation

organic matter, and stabilization of soil pH, without causing excessive salinity. The balanced improvement of these key soil parameters indicates a synergistic interaction between the indigenous PGPR and humic-rich leonardite, promoting nutrient mobilization, microbial activity, and sustainable soil recovery in post-mining or nutrient-poor conditions.

Overall, co-application of R4-3 and 25% leonardite improved both plant biomass and soil nutrient status. Unlike many PGPR that perform under specific crop-soil systems, R4-3 exhibited broad functionality in marginal soils enriched with organic amendments. Previous studies reported high expression of individual PGP traits (e.g., IAA or P solubilization), but their translation to soil improvement was not examined in detail (Chowdhury et al., 2024; Dejtisakdi et al., 2024). This study bridges that gap by linking multiple traits to both plant and soil fertility. This dual benefit highlights the potential of site-adapted PGPR combined with organic soil amendments as a practical approach for soil rehabilitation in post-mining landscapes. This research supports the use of native PGPR strains adapted to specific environments (Akimbekov et al., 2020; AbuQamar et al., 2024). Unlike commercial biofertilizers, R4-3 performed effectively in mining-amended substrates, showing its adaptability and efficiency. These results contribute to the broader concept of microbial precision agriculture, emphasizing the deployment of locally adapted PGPR strains for optimized crop and soil restoration (Pudelko et al., 2021; Oubohssaine et al., 2022; Costa et al., 2023). Although this study demonstrated the effectiveness of R4-3

under controlled pot conditions, further field trials are necessary to validate its performance across different soil types, climates, and cropping systems. Additionally, potential phytotoxic effects observed at higher leonardite concentrations warrant further investigation.

4. Conclusions

This study demonstrated that *Microbacterium maritopicum* strain R4-3, isolated from leonardite-associated rhizosphere in a lignite mine, possessed strong plant growth-promoting traits and outperformed two commercial biofertilizers in promoting lettuce growth. Under both laboratory and pot conditions, R4-3 significantly increased plant biomass, especially when applied in combination with 25% leonardite. In addition to enhancing crop performance, R4-3 improved key soil fertility parameters, including total nitrogen, exchangeable potassium, and organic matter, while contributing to pH buffering under amended conditions. These results emphasize its dual role as both a plant growth enhancer and a soil-restoring agent. The observed synergistic interaction between R4-3 and leonardite underscores the importance of site-specific inoculant strategies for sustainable vegetable cultivation in degraded or nutrient-poor soils. Overall, this approach offers a practical and low-cost solution aligned with organic and low-input farming systems. Further field validation is encouraged to confirm its robustness across diverse soil types and to extend its application in land rehabilitation and precision microbial agriculture.

5. Acknowledgements

This research was supported by the Joint Research and Development Program of the Electricity Generating Authority of Thailand (EGAT) and the Thailand Research Fund (TRF), now under Thailand Science Research and Innovation (TSRI), Project No. RDG61D0010 (2018). The authors thank the Faculty of Sciences and Liberal Arts, Rajamangala University of Technology Isan for providing instruments.

6. Authors' Contributions

Rasapirose Somwatcharajit: performed research, analyzed data, writing original draft; Suchonma Sookruksawong: performed research and analyzed data; Janpen Prakamhang: funding acquisition, design research, performed research, analyzed data, writing original draft, reviewing and editing.

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7. Conflicts of Interest

The authors declare no conflicts of interest relevant to this research.

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