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Bacterial Inoculation and Hydroseeding Enhance Establishment of Revegetation Species on Nickel Mine Overburden: A Contribution to SDG 15

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Abstract: Nickel (Ni) is the most widely produced mining commodity in Indonesia, but mining activities often leave soils with severe physical and chemical limitations that hinder plant establishment and frequently cause revegetation failure during the early growth phase. Hydroseeding and Plant Growth Promoting Rhizobacteria (PGPR) have been studied as approaches for post-mining land reclamation; however, their combined application across different types of Ni overburden has been rarely evaluated. This study aimed to analyze the interaction between planting methods, bacterial inoculation, and overburden types on the germination index and early leaf formation (3–14 days after sowing). The experiment was conducted through a laboratory-scale early growth test using a completely randomized factorial design, and data were analyzed using Analysis of Variance (ANOVA). The results showed that the interaction between bacterial inoculation and application method consistently influenced the germination rate of *Cynodon dactylon*. In contrast, the planting method independently played a dominant role in the germination index of *Crotalaria juncea* and in the number of leaves of both species. The results showed that the interaction between bacterial inoculation and application method consistently affected the germination rate of *C. dactylon*. Conversely, the planting method independently played a dominant role in the germination index of *C. juncea* and the number of leaves of both species. Hydroseeding increased the germination by 186.41 – 7062.5% (in *C. dactylon*) and 15 – 3688% (in *C. juncea*). Bacterial inoculation was unable to increase the germination of *C. dactylon*, but it was able to increase the germination of *C. juncea* by 7.5 – 650% compared to that without inoculation. These findings demonstrate that integrating hydroseeding techniques with microbial inoculation can enhance early plant establishment on Ni mine overburden and support ecological restoration efforts aligned with Sustainable Development Goal 15 (Life on Land). Therefore, it is recommended that revegetation programs in post-Ni mining areas integrate hydroseeding with microbial inoculation and promote community-based restoration initiatives to improve early vegetation establishment and long-term ecosystem recovery.

Keywords: Early establishment; Germination; Hydroseeding; Inoculation; Nickel overburden

Introduction

Nickel (Ni) is the most widely produced mining product in Indonesia, at 35.5 million metric tons. The large reserves of Ni in Southeast Sulawesi make Ni a major mining commodity (Adidharma et al., 2023). However, Ni mining leaves the soil with low levels of essential nutrients, cation exchange capacity, and

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organic carbon, as well as various trace metal residues that ultimately inhibit plant growth (Pędziwiatr et al., 2018; Prematuri et al., 2020). Therefore, the initial phase of vegetation establishment is the most critical period for successful revegetation and soil stabilization, as failure at this stage often results in low long-term rehabilitation success (Lauman et al., 2026). Land reclamation needs to be carried out to improve environmental and ecosystem conditions. Sustainable land reclamation is increasingly framed within the broader agenda of the United Nations Sustainable Development Goals, particularly Sustainable Development Goal 15 (Life on Land). Post-mining land reclamation is one of the major and complex challenges in efforts to restore ecosystem functions to a safe, stable, and pollution-free state (Hendrychová et al., 2020; Suwardi & Randrikasari, 2023).

Cultivation techniques such as hydroseeding have been used as one approach to post-mining land reclamation due to their ability to provide faster vegetation cover and reduce erosion (Clemente et al., 2016; Kolkos & Stergiadou, 2022). Hydroseeding is a planting technique that uses a mixture of seeds, adhesives, fertilizers, and water, which is then sprayed onto the land, thereby supporting better seed distribution and moisture retention in unstable planting media such as mine overburden (Mulyani, 2018). Studies show that hydroseeding can increase the success of post-mining land revegetation. The composition of the materials used in this seeding can improve soil properties and increase germination, as well as stimulate the growth of microbial communities that are beneficial to plant growth (Anshari et al., 2024; Lauman et al., 2026).

In addition, biological approaches, such as Plant Growth Promoting Rhizobacteria (PGPR), are widely used to support plant growth in marginal lands. PGPR are bacteria that live in the rhizosphere of plants and enhance plant growth through various mechanisms, including increased nutrient availability, production of plant hormones, and tolerance to environmental stresses such as heavy metals and other abiotic stressors (R. Ustiatik et al., 2022). A comprehensive review shows that PGPR plays a significant role in improving plant growth and altering heavy metal pathways in soil, as well as modulating root physiology and increasing nutrient uptake. The multifunctional role of PGPR, including nutrient provision, phytohormone production, metal detoxification, and resistance under heavy metal stress, makes it an important component in bioremediation for the revegetation of degraded land (Gupta et al., 2024; Oubohssaine et al., 2025).

Although the effectiveness of hydroseeding and PGPR methods in soil improvement and land revegetation has been documented in various studies, studies that simultaneously evaluate the interaction between seed application techniques (hydroseeding), microbial inoculation, and overburden media characteristics in a single factorial experimental design are still relatively limited. Studies evaluating hydroseeding have demonstrated successful seed distribution and the ability to reduce erosion and enhance soil microbiota in post-mining areas, including based on the microbial diversity detected in hydroseeding revegetation areas compared to conventional methods. This indicates that application techniques influence biotic conditions in revegetated soils (Kirmi & Masyhuri, 2020; Anshari et al., 2024; Ministry of Public Works, 2018).

Meanwhile, research shows that the combination of organic-mineral amendments and PGPR inoculation can improve the physical, chemical, and biological properties of tailings and support plant establishment in highly degraded media through better plant growth in the combination treatment compared to the single treatment (Benidire et al., 2021). However, the majority of studies focus on general vegetation growth responses and medium- to long-term outcomes, such as improvements in soil chemical properties after several months or years of revegetation (e.g., improvements in pH and soil microbial activity, increases in organic carbon and essential nutrients, and reductions in metals in older revegetated areas) (Widjaja & Suryaningtyas, 2024).

Conversely, analysis of plant response dynamics in the early establishment phase, including how hydroseeding, inoculation, and overburden media type interactions affect the germination and leaf formation phases, is still rarely analyzed in reclamation studies temporally and integrated into a comprehensive factorial model. A systematic review of post-mining revegetation also shows that the majority of interventions tested in the scientific literature involve the addition of amendments or fertilizers, with only a small proportion evaluating combinations of treatments including microbial inoculation, and with a primary focus on general vegetation growth parameters rather than early establishment (Navarro-Ramos et al., 2022). Therefore, this study was designed to analyze the effect of bacterial inoculation and different application methods on germination and leaf formation in the early growth phase (3-14 days after planting) by comparing two pioneer plants from different families (*Cynodon dactylon* and *Crotalaria juncea*). This study also compared plant responses to two different nickel overburdens. The results of this study are expected to contribute to the development of revegetation strategies based on an integrated technical and biological approach in extreme media conditions.

Method

Soil Sampling

Soil samples for the experiment were taken from the WIUP PT PMS in Pomalaa, Kolaka Regency, Southeast Sulawesi. Geographically, the research location is located at 4°12'20.4"– 4°12'20.4" S and 121°37'19.7"– 121°38'36.8" E. Soil samples were taken from former mining sites that had not been reclaimed from two types of overburdens, namely limonite and saprolite (Fig. 1).

Figure 1. Overburden collection point on the former nickel mining land owned by PT PMS, in Pomalaa, Southeast Sulawesi, for greenhouse-scale planting trials. (Source: Google Earth, accessed on February 23, 2026)

Analysis of Soil Chemical Properties

Analysis of several soil chemical properties was conducted with reference to the Technical Manual for Chemical Analysis by the Soil Research Institute in 2023 (Eviati et al., 2023). The parameters observed included pH (electrometry), organic C (Walkley and Black), total N (Kjeldahl), available P (Bray I), exchangeable K (NH4OAc, pH 7.0), and total Ni content (atomic absorption spectrophotometry, SSA) in the soil.

Seed Germination Bioassay

Germination tests were conducted using *Crotalaria juncea* and *Cynodon dactylon* seeds, according to the methods described by Fahsi et al. (2021); Fiodor et al. (2023), with modifications. Seeds were surface sterilized using 2% sodium hypochlorite for 2 min and 70% ethanol for 1 min, followed by rinsing with sterile distilled water. The seeds were then soaked in a bacterial suspension (10^7 CFU/mL) or sterile distilled water (control) for 2 h. After soaking, seeds were placed on Whatman filter paper saturated with a 1,000 ppm Ni and 0 ppm Ni (sterile distilled water) solution in petri dishes. During incubation, the seeds were supplemented with 5 mL of bacterial suspension (treatment) or sterile distilled water (control). The experiment was performed in triplicate, with 10 seeds per petri dish. Germination was observed for 7 days; seeds were considered germinated if the radicle length reached a minimum of 2 mm. The germination percentage was calculated according to Equation 1 (Sarioa et al., 2022). The treatments consisted of *C. dactylon* and *C. juncea* with and without bacterial consortium.

$$Germination\ rate\ (\%) = \frac{Number\ of\ seeds\ that\ germinated}{Total\ number\ of\ seeds} \times 100 \quad (1)$$

Pot Trial

The planting medium consisted of Ni overburden (OB) materials (limonite and saprolite) obtained from the PT PMS Ni mining area. The experiment was conducted using a completely randomized factorial design with 3 factors (OB type, consortium bacteria inoculation, application method) and 2 levels (with and without). The following treatments: LTH: limonite without hydroseeding and bacterial consortium; LTHB: limonite without hydroseeding + bacterial consortium; LH: limonite hydroseeding without bacterial consortium; LHB: limonite hydroseeding + bacterial consortium; STH: saprolite without hydroseeding and bacterial consortium; STHB: saprolite without hydroseeding + bacterial consortium; SH: saprolite hydroseeding without bacterial consortium; SHB: saprolite hydroseeding + bacterial consortium. Each type of OB was placed in a 30 x 50 cm container. Sterilization was not carried out, so that the overburden conditions remained similar to their original conditions in the field, referring to the research (Barrera et al., 2025). Seeds were selected for viability based on density; only those that sank after soaking in sterile distilled water for 24 h were used (Baiti & Arisoesilaningsih, 2015; Anshari et al., 2023). Seeds were soaked in a bacterial suspension of 10^7 CFU/mL (inoculated treatment) or sterile distilled water (control) for 2 h before planting (Fahsi et al., 2021; Fiodor et al., 2023). A total of 50 mL of the bacterial consortium suspension (10^7 CFU/mL) was added (Tirry et al., 2018; Zhang et al., 2022; Kumar et al., 2023). Manure was applied at a dosage of 5 tons/ha, based on a previous recommendation by Leomo et al. (2021). The application of hydroseeding and maintenance in greenhouses refers to SE Minister of Public Works No. 02/SE/M/2018 (Minister of Public Works, 2018). Hydroseeding is applied manually by pouring hydroseeding slurry onto the overburden surface, then leveling it using a small shovel. Observations were made from the time the shoots began to emerge and continued for 14 Days after Planting (DAP). The observation parameters included the germination index and the average number of leaves.

Statistical Analysis

Data were tabulated using Microsoft Excel and tested for normality using the Shapiro–Wilk test. Data with abnormal distribution were transformed using SQRT. Analysis of variance was performed using Analysis of Variance (ANOVA) at a 5% significance level. Treatments that had a significant effect were then further tested using the Honest Significance Difference test (HSD) at a 5% significance level. Statistical analyses were tested using R software.

Result and Discussion

Chemical Properties of Nickel Overburden

Parameters such as pH, organic C, total N, and available P indicate that the medium has a relatively low level of fertility, except for the pH value, which is classified as neutral (Table 1). This condition differs from other former mines, which generally show acidic pH due to the oxidation of sulfide minerals. The parent material at the PT PMS Ni mine consists of ultramafic rocks that have undergone weathering to form Ni laterite (Raivel & Firman, 2021). The weathering of ultramafic rocks produces soil with a neutral or slightly alkaline pH, around 6.5 – 7.9, due to the rock's high Mg and Fe content (da Silva et al., 2022).

The removal of topsoil and the lack of organic matter input due to mine operations reduce nutrient availability and microbial activity (Bhattacharyya et al., 2022; Prematuri et al., 2020; Ustiatik et al., 2024). These conditions potentially limit early physiological processes in seeds, particularly during the imbibition and enzymatic activation phases. Total Ni content exceeding normal limits has the potential to cause heavy metal stress. The laterization process in ultramafic rocks causes the dissolution of Ni-Co and other metals, which then accumulate mainly in the limonite and saprolite layers (Schodde & Guj, 2025). Ultramafic soils are characterized by a low Ca/Mg ratio, a deficiency of essential nutrients such as N, P, and K, and high Ni, Cr, and Co content, which causes stress on plants (Pędziwiatr et al., 2018).

Table 1. Basic Chemical Properties of Two Types of Ni Overburden

Parameter	Limonite	Saprolite
Ph	7.13 (N)	7.36 (N)
Organic carbon (%)	0.07 (VL)	0.06 (VL)
Total N (%)	0.05 (VL)	0.02 (VL)
Available P (mg/kg)	3.51 (VL)	4.99 (VL)
Exchangeable K (me/100 g)	0.08 (VL)	0.08 (VL)
Ni-total (mg/kg)	8468.69 (EN)	12337.22 (EN)

Notes: EN: exceeds normal limits; N: neutral; VL: very low. Criteria based on the Technical Guide for Chemical Analysis by the Soil Research Institute in 2023 (Eviati et al., 2023).

Plant Germination Response in the Laboratory Scale

The interaction between bacterial inoculation and Ni concentration showed a significant effect in laboratory-scale experiments ($p < 0.05$). Curiously, under Ni-free conditions (0 ppm), bacterial inoculation resulted in a relatively lower germination rate compared to the non-inoculated treatment. However, at high Ni concentrations (1000 ppm), bacterial inoculation significantly increased the germination rate of *C. juncea* to 100% compared to non-inoculation, although in *C. dactylon*, bacterial inoculation showed no difference from the control (non-inoculated) (Fig. 2).

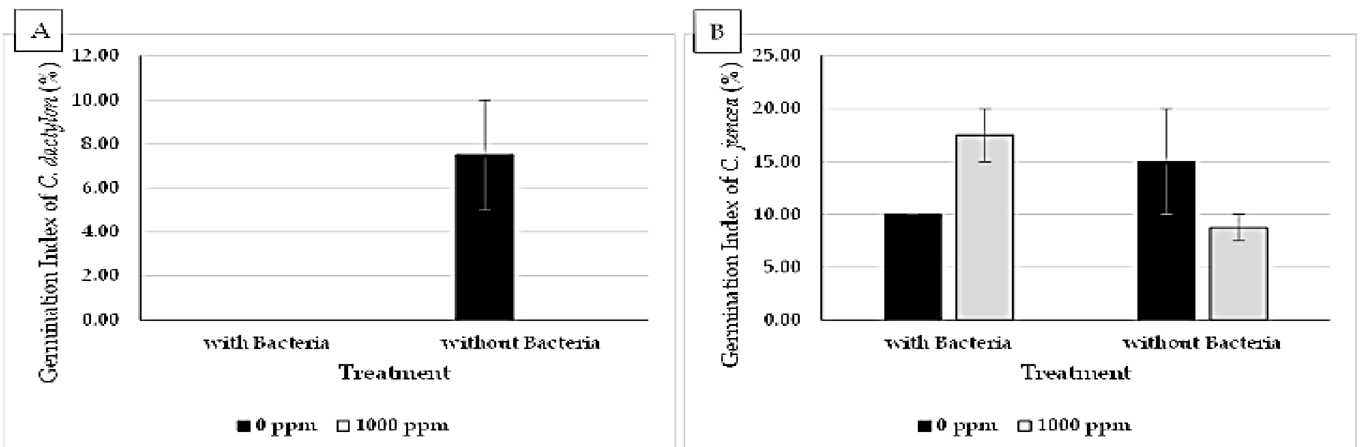

Figure 2. Effect of bacterial inoculation and Ni concentration on the germination index of: A) *Cynodon dactylon* and B) *Crotalaria juncea* on a laboratory scale

This shows that the benefits of bacteria are influenced by the level of heavy metal stress. This finding is also in line with previous reports that bacteria tend to provide better growth stimulation under stressed conditions (Backer et al., 2018). Under stressed conditions, bacterial inoculation was able to reduce the toxic effects of Ni through biosorption and the reduction of heavy metal cations into less toxic forms. In addition, the ability of bacteria to produce phytohormones was also able to increase plant tolerance to stress, thereby enhancing their growth under environmental stress (Pishchik et al., 2021).

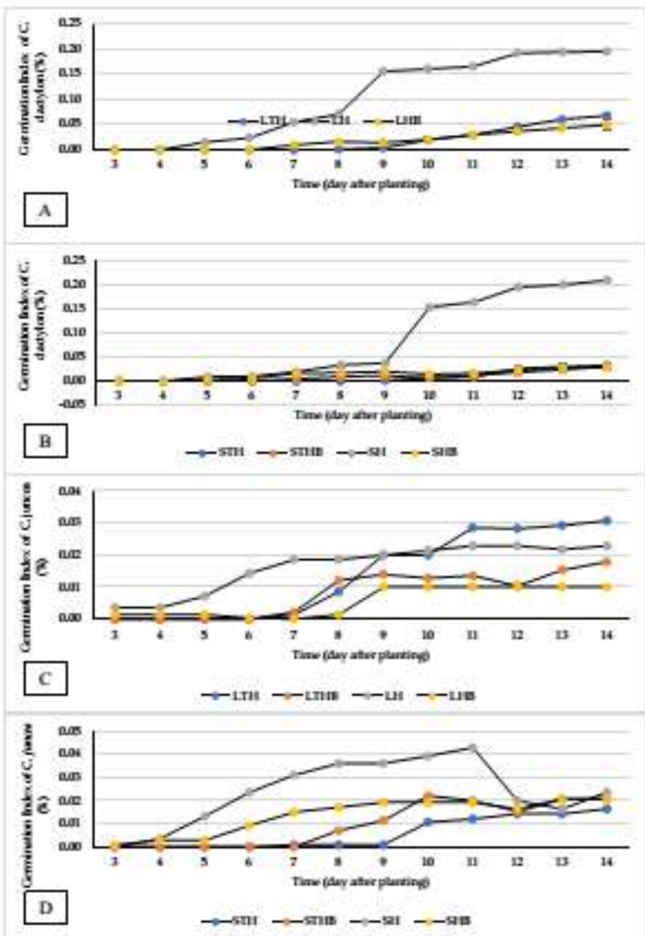

Figure 3. Effect of bacterial inoculation and application methods on the germination index of: A) *Cynodon dactylon* with limonite overburden; B) *Cynodon dactylon* with saprolite overburden, C) *Crotalaria juncea* with limonite overburden; D) *Crotalaria juncea* with saprolite overburden on a greenhouse scale. Treatments: LTH: limonite without hydroseeding and bacterial consortium; LTHB: limonite without hydroseeding + bacterial consortium; LH: limonite hydroseeding without bacterial consortium; LHB: limonite hydroseeding + bacterial consortium; STH: saprolite without hydroseeding and bacterial consortium; STHB: saprolite without hydroseeding + bacterial consortium; SH: saprolite hydroseeding without bacterial consortium; SHB: saprolite hydroseeding + bacterial consortium

The difference in germination index values between *C. juncea* and *C. dactylon* shows that plant species respond differently to bacterial inoculation. Specific plant-microbe interactions contribute to PGPR performance. Differences in plant species, or even cultivars within a species, can show varying responses to the same PGPR strain because host specificity produces different physiological responses, including plant growth and disease resistance (Zhou et al., 2016; Yang et al., 2024). The success of PGPR inoculation depends on the interaction between the host plant and bacteria, which is mediated through root exudates. Primary metabolites such as organic acids, glucose, and amino acids present in root exudates are utilized as substrates by microbes in the rhizosphere. Meanwhile, secondary metabolites such as flavonoids, coumarins, benzoxazinoids, strigolactones, triterpenes, ethylene, and others influence the composition of microbes in the rhizosphere (Kong & Liu, 2022).

Plant Germination Response in the Greenhouse Scale

Greenhouse experiments using three factors (type of overburden, bacterial inoculation, and application method) showed varying effects during the observation period on both pioneer plants, *C. dactylon* and *C. juncea*. The germination rate was observed in the range of 3 – 14 DAP (days after planting) (Fig. 3). The interaction between inoculation and application method had a significant effect on *C. dactylon* germination at 5 – 14 DAP. This was due to the interaction between the two factors, which created ecological conditions suitable for germination. Bacteria as PGPR help increase nutrient availability through N fixation, P solubilization, and the production of essential plant hormones such as indole acetic acid (IAA), abscisic acid, and cytokinin (Saadaoui et al., 2022; Ustiatik et al., 2022; Chabbi et al., 2024). Hydroseeding provides better contact between the inoculant and the seeds, resulting in more optimal bacterial colonization on the surface. Hydroseeding also improves early vegetation establishment in several mine reclamation studies, including field-scale experiments. Results show that areas treated with hydroseeding exhibit improved germination and seedling growth compared to areas without hydroseeding (Anshari et al., 2024; Lauman et al., 2026).

C. juncea showed different germination responses during the observation period of 3 – 14 DAP. The method affected the germination rate of *C. juncea* at 3 – 5 DAP, while at 6 DAP, interactions between inoculation and method began to appear. However, at 7 – 9 DAP, the germination index of *C. juncea* was again affected by the application method ($p < 0.05$). Hydroseeding was able to increase the germination response by 15 – 3688% compared to without hydroseeding. Meanwhile, bacterial inoculation was able to increase germination by 7.5 – 650% compared to that without inoculation.

Hydroseeding provides a slurry consisting of mulch and soil nutrients, which can maintain moisture and increase seed contact with the medium more effectively than manual planting (without hydroseeding), thereby accelerating the initiation of germination (Parsakhoo et al., 2018; Verma et al., 2024). Other studies have also shown the success of hydroseeding in improving plant growth and survival rates as well as stabilizing microhabitats on former coal mining sites. Hydroseeding can accelerate vegetation growth, which in turn promotes faster ecological recovery (Yuningsih et al., 2021; Anshari et al., 2023). The difference in the effect of inoculation on germination response in the two species shows that PGPR inoculation varies greatly depending on the bacterial and plant species, soil type, inoculum density, and environmental conditions. In addition, the inoculated bacteria must also be able to survive in the soil, be compatible with the inoculated plants, and interact with native soil microbes and existing abiotic factors (Saadaoui et al., 2022).

Lower germination index values in greenhouse experiments using Ni overburden compared to laboratory tests using sterile filter paper due to more heterogeneous microenvironmental conditions. Ni overburden has poor soil structure and porosity, resulting in very low water retention capacity. This causes seed inhibition to be inhibited, leading to a decrease in the germination rate. Water is necessary for enzymatic reactions and the mobilization of seed storage reserves, including lipids, proteins, and carbohydrates (Sghaier et al., 2022). In addition, higher temperatures in greenhouses can also interfere with seed germination, during the study period, the temperature at greenhouse was 40-45 °C. Temperature also affects the inhibition process (thermo-inhibition), where studies show that butterfly pea (*Clitoria ternatea*) germination is optimal at 24 – 28 °C and decreases dramatically at sub-optimal temperatures of 36 – 40 °C (Campbell et al., 2020). Other studies also show that ryegrass (*Lolium perenne* L.) germination shows optimal growth at 25 °C and declines rapidly at 35 °C. Osmotic potential also completely inhibits seed germination at -1.0 MPa (Javaid et al., 2022).

High metal levels also cause a decrease in the germination index in greenhouse experiments. Ni toxicity inhibits plant metabolism, causing cell damage and even death. Ni can replace some molecules in plants, block important functional groups in biological molecules, and alter enzymes (Jan et al., 2019). Ni can remove Ca from bond complexes and replace Mg in chlorophyll, thereby inhibiting electron transport and reducing energy supply (Hassan et al., 2019). High Ni concentrations during the vegetative growth period cause stunted shoot and root growth and disrupt branch development (Khan et al., 2023). Ni inhibits seed germination and seedling growth through changes in hydrolytic enzyme activity, followed by inhibition of food reserve transport from the endosperm to the embryonic axis (Pishchik et al., 2021).

Leaf Number Response in the Greenhouse Scale

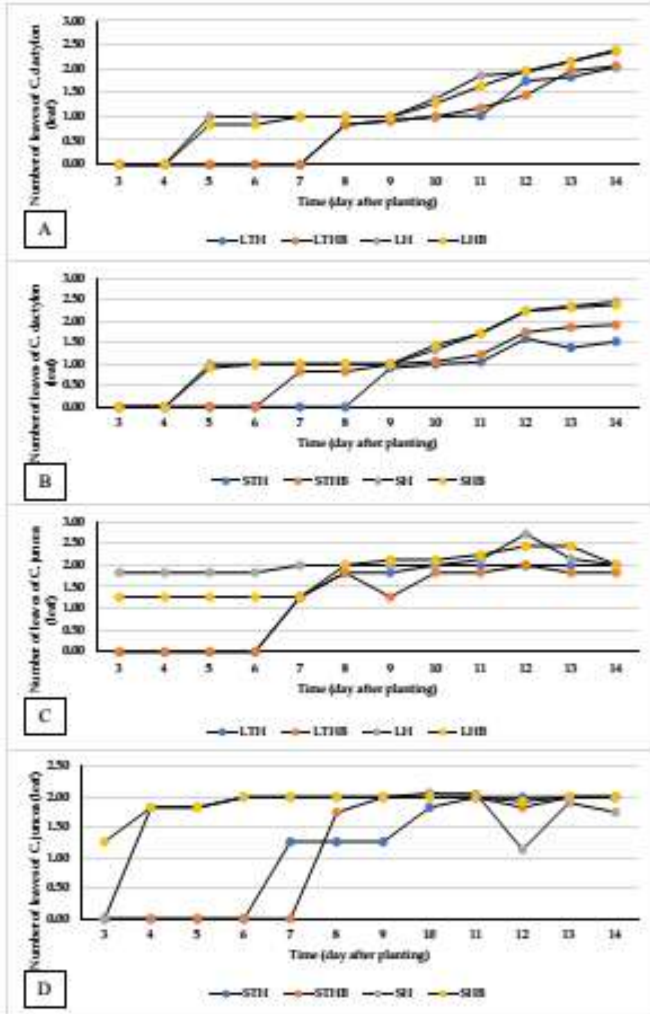

Figure 4. Effect of bacterial inoculation and application methods on the number of leaves of: A) *Cynodon dactylon* with limonite overburden; B) *Cynodon dactylon* with saprolite overburden, C) *Crotalaria juncea* with limonite overburden; D) *Crotalaria juncea* with saprolite overburden on a greenhouse scale. Treatments: LTH: limonite without hydroseeding and bacterial consortium; LTHB: limonite without hydroseeding + bacterial consortium; LH: limonite hydroseeding without bacterial consortium; LHB: limonite hydroseeding + bacterial consortium; STH: saprolite without hydroseeding and bacterial consortium; STHB: saprolite without hydroseeding + bacterial consortium; SH: saprolite hydroseeding without bacterial consortium; SHB: saprolite hydroseeding + bacterial consortium

The number of leaves was observed at 3 – 14 DAP as an indicator of early vegetative growth (Fig. 4). The results of the analysis of variance showed that the number of leaves of *C. dactylon* plants was significantly affected by the method during the 6 – 14 DAP observation period ($p < 0.05$). Meanwhile, in *C. juncea*, the method significantly affected the number of leaves during the observation period of 3 – 8 DAP ($p < 0.05$) and then lost its effect in subsequent observations ($p > 0.05$). Hydroseeding was able to create a more stable microhabitat. Hydroseeding mulch can be a medium for the germination and growth of pioneer plant seeds in former coal mining areas, especially for the Leguminosae and Poaceae families (Anshari et al., 2018). Other studies have also shown that hydroseeding mulch can increase plant morphological traits and biomass in the short term through better modification of the microenvironment, including maintaining moisture around the seeds. This study also found that Legume species produced higher growth (including number of leaves) and dry weight with hydroseeding application (Gudyniene et al., 2021).

The difference in response between the two plants may be due to differences in their responses to environmental stress. Plants respond to environmental stress through the transcription of several stress-related genes that experience increased or decreased expression, resulting in a reaction to stimuli. Research shows that *C. dactylon* responds to abiotic stress by decreasing vertical growth, plant height, stolon length, leaf length and width, and the number of tillers to reduce the rate of evapotranspiration. This grass also contains enzymatic antioxidants such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), as well as non-enzymatic antioxidants such as carotenoids and flavonoids, which play an important role in reactive oxygen species (ROS) stability, thereby protecting plants from oxidative damage (Noor et al., 2024). Meanwhile, in *C. juncea*, leaf growth is known to be very sensitive to environmental stress. Research shows that an increase in heavy metals significantly reduces the number of leaves. This indicates physiological limitations in maintaining vegetative growth in degraded soil conditions. In addition, the number of *C. juncea* leaves tends to reach a maximum value in the early stages of growth and decreases as the growth phase progresses (Maldonado-Peralta et al., 2022; dos Santos et al., 2024).

Implications for Land Reclamation and Sustainable Development

The findings of this study have important implications for the management of post-mining land reclamation, particularly in Ni mining areas where overburden materials often exhibit poor chemical properties that limit plant establishment. The results indicate that integrating hydroseeding techniques with bacterial inoculation can improve early plant establishment, which is a critical stage for successful revegetation and soil stabilization. Faster and more uniform germination can accelerate vegetation cover formation, thereby reduce erosion risk and enhancing the recovery of soil biological activity in degraded substrates. From a practical perspective, the application of hydroseeding combined with beneficial microbial inoculants serves as an efficient strategy for large-scale revegetation in post-mining landscapes. Furthermore, sustainable reclamation practices that restore vegetation cover and ecosystem functions contribute to achieving Sustainable Development Goal 15 (Life on Land), which emphasizes land restoration and biodiversity conservation.

Conclusion

Bacterial inoculation affected the germination index on a laboratory scale and increased the germination of C. juncea to 100% at a Ni concentration of 1.000 ppm. The interaction between bacterial inoculation and application method only affected the germination of *C. dactylon*, while the germination of *C. juncea* and the number of leaves in both species were affected by the method. The type of overburden did not affect the germination or number of leaves in either species. Hydroseeding was able to increase the germination response by 0.63 – 78.75% compared to without hydroseeding. Meanwhile, bacterial inoculation was able to increase germination by 4.17 – 30.81% compared to that without inoculation. Overall, the hydroseeding method was the most consistent factor influencing the initial growth success of both species, while the effectiveness of bacterial inoculation was highly contextual and dependent on the combination of application methods and overburden type. These findings indicate that optimizing application techniques is more crucial than administering a single inoculant in the early phase of post-nickel mining land revegetation. Further evaluation of growth, biomass, and changes in soil chemical properties has not been conducted, so further research is needed to assess the long-term impact of the treatment combinations.

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Author Contributions
Conceptualization, A.P.A. and R.U.; methodology, A.P.A. and R.U.; validation, K.S.W. and M.Y.; formal analysis, A.P.A; data curation, A.P.A.; writing—original draft preparation, A.P.A,

R.U., M.Y.; review and editing, K.S.W. and J.W; funding acquisition, R.U. and J.W. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest
The authors declare no conflict of interest.

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