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Evaluating Microbial Biofertilizers for Root Colonization Potential in Narra (*Pterocarpus indicus* Willd.) and Their Efficacy in Heavy Metal Remediation

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Abstract: Bioremediation technology, another strategy known for restoring degraded environments, utilizes beneficial microorganisms, including arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing bacteria (NFB). Despite its potential, the biological processes of these microorganisms in contaminated sites remain poorly understood, hindering effective pollutant toxicity reduction. Establishing a connection between plant root systems and these microorganisms is crucial for enabling plant survival in heavy metal-contaminated soils. Narra (*Pterocarpus indicus* Willd.), a leguminous plant, typically associates with symbiotic nitrogen-fixing bacteria, forming nodules in the roots. Additionally, Narra forms a symbiotic relationship with AMF, phosphorus-fixing microbes, making it an ideal tree species for rehabilitating mined-out areas. In this study, five microbial biofertilizers, namely: MYKORICH®, MYKOVAM®, newMYC, newNFB, and combined newMYC+newNFB, plus a control were used to test their root colonization potential on Narra seedlings grown in nickel (Ni) and gold (Au) mined-out soils collected from Taganito Mining Corporation (TMC) and Manila Mining Corporation (MMC) in Claver and Placer, Surigao del Norte, Philippines, respectively. The results showed that newMYC had the highest root colonization in Ni mined-out soil, while MYKORICH® excelled in Au mined-out soil. The AMF spore count was highest in MYKORICH® for Ni mined-out soil and newMYC in Au mined-out soil. NFB colonization was highest in newMYC-treated seedlings for Ni mined-out soil and combined newMYC+newNFB for Au mined-out soil. The microbial biofertilizers utilized in this research, such as MY-KORICH®, MYKOVAM, newMYC, newNFB, and combined newNFB and newMYC, naturally occur in the environment and can be easily extracted. This cost-effective characteristic provides an advantage for mining companies seeking treatments for soil amelioration to rehabilitate mined-out areas.

Keywords: bioremediation technology; arbuscular mycorrhizal fungi; nitrogen-fixing bacteria; *Pterocarpus indicus*

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1. Introduction

Understanding the relationship between plants and microorganisms provides a sound basis for the mechanisms behind harnessing critical biological processes necessary for the soil cleanup of heavily contaminated mined-out areas [1]. These microorganisms naturally occur in productive soils and play an essential role in nutrient transformations to provide sufficient nutrients to plants and other organisms living in the soil. Soil microbes hold various enzymes that play crucial roles in soil nutrient transformations, especially nitrogen and phosphorus, the major plant nutrients derived from the soil [2]. In this regard, understanding the interaction between plants and microorganisms in heavily contaminated soils is crucial. This interaction serves as an important mechanism to assess the effective removal of heavy metals from the soil while enabling plants to grow vigorously and luxuriously. Such growth is essential for the successful re-vegetation of formerly mined-out areas. Furthermore, the technology of using microbes to control environmental pollution is promising. Bacteria and other microorganisms offer a valuable platform for an improved bioremediation model of many environmental pollutants [3].

Bioremediation is a widely accepted alternative for cleaning the environment [4], and in situ bioremediation technologies have high public acceptance in developed economies (North America and Europe). In contrast, ex-situ bioremediation technologies are used more in developing regions [5]. In the Philippines, a bioremediation method that uses microorganisms was used, with the most with notable successes [6]. An example of a successful rehabilitation using this technique is the copper mined-out area in Mogpog, Marinduque, where a plant survival rate of 95% was achieved [7]. Before rehabilitating the said mined-out area, it was barren and abandoned due to harsh soil conditions that hindered the rooting of the plant species [8]. Furthermore, rehabilitation success is often judged based on plants' survival or growth rate [9], selection of suitable restoration techniques, excessive heavy metal content, poor physical structure, and inadequate soil nutrition [10]. Notably, the root colonization potential of Narra seedlings using biofertilizer inoculation has yet to be explored, as demonstrated by other studies with other plant species [11–15].

Phytoremediation is one of the recommended mechanisms for the bioremediation of mined-out areas [16]. Phytoremediation is defined as a bioremediation strategy that uses plants and their physical, chemical, biological, and microbiological processes in contaminated sites to minimize the toxicity of pollutants. This method is a low-cost and environmentally friendly technology that involves not only the degradation of pollutants but also the removal of the pollutants from the environment without causing further degradation (Broda, 1992) as cited by [17]. In the study of [18], there were four (4) tree species, namely Ipil-ipil (*Leucaena leucocephala* Linn.), Narra (*Pterocarpus indicus* Willd.), Mansanitas (*Muntingia calabura* Linn.), and Calumpit (*Terminalia macrocarpa* Steud.) which exhibited high percentage survival and excellent growth performance in nickel mined-out areas. *Pterocarpus indicus* Willd., *Acacia mangium* Willd., and *Eucalyptus urophylla* S.T. Blake, with microbial biofertilizers and applied with compost and lime, showed significantly higher survival and growth compared to the previously planted seedlings that showed a low survival rate, stunted growth, and unhealthy seedlings as indicated by the yellowing leaves of the plants [15]. Despite the promising effects of these microbial biofertilizers, the results reveal that the different treatments had varying effects on plant growth and were only applied in the Cu-rich soil of Mogpog, Marinduque.

This research focused on nickel and gold mined-out areas situated in Mindanao. This selection was intentional as the objective was to develop bioremediation protocols tailored to the unique conditions of mined-out areas in the Philippines. Further, this study utilized the Narra (*Pterocarpus indicus* Willd.) species due to their affinity for nitrogen-fixing bacteria (NFB) and their distinctive capacity to establish a symbiotic relationship with microorganisms. Narra seedlings form root nodules associated with nitrogen-fixing bacteria (NFB) and establish connections with mycorrhizal fungi, which are phosphorus-fixing microbes [19]. As a result, Narra seedlings hold so much potential as a bioremediation species in rehabilitating mined-out areas by creating favorable growth conditions in the rhizosphere that increase their survivability in the harsh environment of mined-out areas. The results of this study are essential to developing site-specific protocols for the bioremediation of mined-out areas in the Philippines.

2. Materials and Methods

2.1. Experimental Design

Two concurrent nursery experimental set-ups (see Figure 1) were conducted following a randomized complete block design (RCBD) since this set-up emulates the mined-out area conditions while testing the effectiveness of different treatments on Narra seedlings. The experimental design minimizes variability within a replicate by randomly arranging the treatments in a block. This experiment used ten blocks with six treatments for each block. Different colors represent different treatments; there are ten blocks (1–10) and six treatments [A-control, B-MYKORICH®, C-MYKOVAM®, D-newMYC, E-newNFB, and F-combined newMYC+newNFB] (Please note: each treatment was replicated five times).

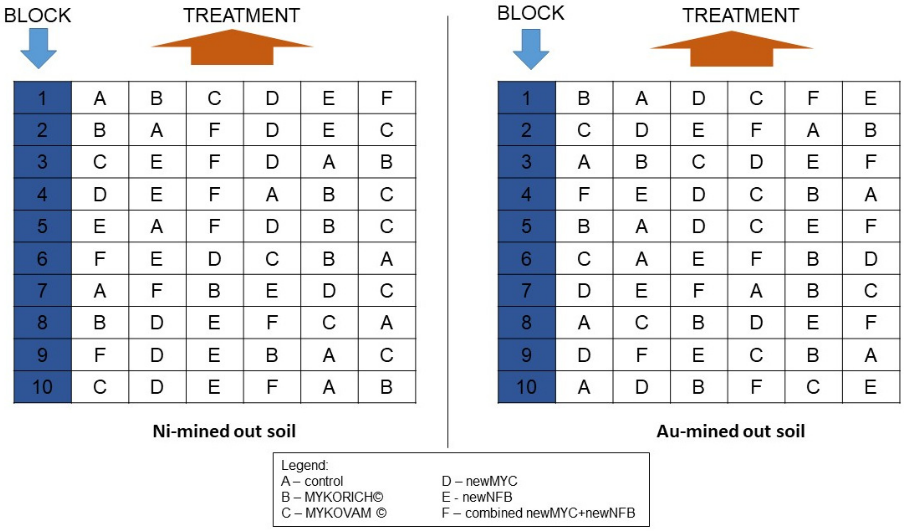


Figure 1. RCBD nursery experimental layout.

2.2. Soil Collection, Processing and Characterization

There were two (2) mined-out soils used in this study, Ni and Au mined-out soils, which were collected in the mining sites of Taganito Mining Corporation (TMC), Claver, Surigao del Norte, and Manila Mining Corporation (MMC), Placer, Surigao del Norte, Philippines, respectively. A total of 400 kilos of mined-out soil were collected from each site. The collected soils from TMC and MMC were independently pooled, air-dried, and sieved using a $0.5' \times 0.5'$ metal wire mesh to remove unwanted debris and to collect finer soil particles. The sieved soils were homogenized by mixing thoroughly by hand. After the sieving process, the soils were mixed with compost at a 1:1 ratio. The composite soils were then potted into $6' \times 10'$ polyethylene bags, which were used to repot the Narra seedlings one month after inoculating the biofertilizers mentioned previously. A total of 600 potted soils in $6' \times 10'$ polyethylene bags were prepared (where 300 potted polybags for each mined-out soil).

Before inoculating these microbial biofertilizers, a pre-assessment of soil nutrient status was conducted. The results of the pre-assessed collected mined-out soils, as shown in Table 1, TMC soils have deficient-to-low nutrient availability (% organic matter, total nitrogen, available P, and exchangeable K) and have a high nickel (7166 ppm) content, which is an order of magnitude higher than the Dutch standard intervention value of 210 ppm.

On the other hand, MMC soils have low-to-moderate nutrient availability and a pH value of 6.14. Additionally, MMC soils have high concentrations of molybdenum and copper, of 39,400 ppm and 449.2 ppm, respectively, which exceed the Dutch standard intervention value of 200 ppm (molybdenum) and 190 ppm (copper), respectively (Table 1). These values indicate severe contamination of the soils, and immediate remediation is highly recommended.

Table 1. TMC and MMC mined-out soil characterization.

Parameters	Mined-Out Soils	
	TMC (Ni)	MMC (Au)
% Organic matter	0.4 (low)	0.43 (low)
pH	7.17	6.14 (slightly acid)
Available P (ppm)	1.3 (low)	7 (moderately high)
Exchangeable K (ppm)	6.0 (deficient)	77 (moderately sufficient)
Total N (%)	-	0.048
Electrical conductivity (μS/cm)	113.3	837.4
Iron (ppm)	326,600	-
Nickel (ppm)	7166.66	-
Copper (ppm)	-	472.1
Manganese (ppm)	-	449.2
Molybdenum (ppm)	-	39,400

These indicators were given by the Regional Soil Laboratory, Department of Agriculture, Butuan City.

2.3. Inoculation of Seedlings

The collected and prepared Narra seedlings were allowed to grow naturally in a shaded area to recover from stress during the transport from the Department of Environment and Natural Resources (DENR)-Provincial Environment and Natural Resources Office (PENRO) Nursery in Zamboanga del Norte to the Forest and Wetland Research, Development and Extension Center—Ecosystems Research and Development Bureau (FWRDEC-ERDB) Satellite Office in Butuan, Agusan del Norte. After two weeks, Narra seedlings were inoculated with the various formulations of the biofertilizers by making two holes (2–3 inches depth) in the pot using a barbecue stick.

The following treatments were used during the inoculation of Narra seedlings (Table 2).

Table 2. Composition of each microbial biofertilizer and its mode of appplication.

	Treatment	Mode of Application/Inoculation
1	Control (no inoculation but applied with complete fertilizer)	No inoculation
2	MYKORICH® (composed of 12 species of AMF in a capsule)	Four capsules were inserted in the holes (2–3 inches depth) of the seedling
3	MYKOVAM® (composed of 12 species of AMF in the sand substrate)	10 g was placed in the holes (5 g on each hole)
4	newMYC (composed of 5 high sporulating AMF isolated from the rhizosphere of plants growing in the mine tailing areas of Barangay Capayang, Mogpog, Marinduque)	10 g was placed in the holes (5 g on each hole)
5	newNFB (composed of 5 fast-growing NFBs isolated from the rhizosphere of plants growing in the mine tailing areas of Barangay Capayang, Mogpog, Marinduque)	10 g was placed in the holes (5 g on each hole)
6	Combined newMYC+newNFB (combination of Mogpog AMF and NFB isolates)	10 g was placed in the holes (5 g on each hole)

The treated Narra seedlings were grown for another month in the established nursery at the FWRDEC Satellite Office, Butuan City, Philippines, to allow for the microbes to infect the Narra roots before being transferred into the poly bags filled with composite soils (mined-out soil mixed with compost and complete fertilizer). The experimental seedlings were handled correctly to avoid root disturbance while transplanting into the composite soils.

2.4. Microbial Population Analysis

After six (6) months, three (3) replicates per treatment were collected and processed to assess and analyze the microbial root infection potential of the treatments to the Narra seedlings raised in Ni and Au mined-out soil, respectively. The 18 seedlings from each mined-out soil were brought to the University of the Philippines, Los Baños (UPLB) BIOTECH Laboratory to analyze the mycorrhizal root colonization, spore count, and nitrogen-fixing bacterial population.

The population of NFB in the roots was determined using a Dobereiners' nitrogen-free agar medium [20], where the pH was adjusted to 7.0 using 1M NaOH. The medium was composed of 5.0 g malic acid, 4.0 g KOH, 0.1 g $CaCl_2$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $MnSO_4 \cdot H_2O$, 0.9 g K_2HPO_4, 10 mg $FeSO_4 \cdot 7H_2O$, 5.0 mg $Na_2MoO_4 \cdot 2H_2O$, 3.0 mL Bromothymol blue, 0.5% alcohol solution, and 15.0 g agar. All components were dissolved in 1 L of distilled water, and the pH was adjusted to 7.0 using 1 M NaOH. The roots were first cleaned under running water, surface sterilized with Clorox, and washed several times with sterile water. The roots were then macerated in a mortar and pestle, where dilutions were prepared. Aliquots were streaked on agar medium. Colonies were counted.

Mycorrhizal root colonization was assessed following the procedure of [21] where fine root samples (0.2 g plant-1 fine roots (<0.2 mm diameter)) were cleared with 10% KOH in a water bath set at 90 °C for 30 min and then cooled at room temperature and bleached overnight with 50% H_2O_2. After that, the H_2O_2 was replaced with 0.1 N HCl, washed in water, and stained in 0.05% methylene blue with 70% glycerine for 30 min in a water bath at 90 °C. Excess stain was removed using tap water. Stained roots were mounted on 50% glycerine and observed under a dissecting microscope. Gridline intersect methods described by [21] were used to determine root colonization. All mycorrhizal and non-mycorrhizal roots that crossed the grid lines viewed in ten field views (under the microscope) were counted. Roots with vesicles (round to oval in shape and with dark blue color), hyphae, or spores were counted as mycorrhizal colonized roots. Percent colonization (in each field view at 40× magnification) was computed as the total roots minus the colonized ones divided by the total counted roots and multiplied by 100.

2.5. Statistical Analysis

All data gathered were subjected to a one-way ANOVA procedure using IBM SPSS Statistics 22®. When all the treatment means were rendered significant, mean separation was performed using the Least Square Difference (LSD) method at a 95% significance level. Also, Pearson's correlation coefficient was used to determine the relationship of the root colonization of the applied biofertilizers to the growth of Narra.

3. Results

3.1. Soil Nutrient Status after Six (6) Months

The results of the soil analysis after six (6) months of the experiment are presented in Table 3 below. Comparisons can be drawn from Table 1, revealing an increase in percentages of organic matter (%OM), total nitrogen (% Total N), and available phosphorus, along with a significant decrease in heavy metal elements like iron and nickel in the Ni mined-out soil. In relation to the application of biofertilizers to soil with heavy metal content, the data indicate that the concentrations of both iron and nickel were highest in the control group soil. Conversely, the soil treated with MYKORICH© exhibited the lowest iron and nickel content in the Narra seedlings. Consequently, for the nickel mined-out soil substrate, MYKORICH© emerged as the most effective treatment for potential heavy metal remediation, as evidenced by the results presented below.

Meanwhile, the results of the soil analysis in the Au mined-out soil after six (6) months are displayed in Table 4 below. Upon comparing with Table 1, it is evident that there is an increase in percentages of organic matter (%OM), total nitrogen (% Total N), and available phosphorus, accompanied by a significant decrease in heavy metal elements present in Au mined-out soil, including copper, manganese, and molybdenum.

Table 3. Ni mined out soil status after six (6) months.

Treatment	TMC (Ni Mined-Out Soil)					
	% Organic Matter	Total N (%)	Available P (ppm)	Exchangeable K (ppm)	Iron (ppm)	Nickel (ppm)
1-control	5.5	0.31	4055.33	1064.67	16.89	4217.33
2-MYKORICH	3.53	0.28	3571.33	1469.67	10.75	2220.33
3-MYKOVAM	4.37	0.25	3599.33	971.33	12.41	2773
4-newNFB	5.7	0.3	3817	1338	13.62	3233
5-newMYC	5.67	0.3	4169	1529.33	11.98	2621
6-combined newNFB+newMYC	5.33	0.28	4046.33	1304	14.45	3431

Table 4. Au mined-out soil status after six (6) months.

Treatment	MMC (Au Mined-Out Soil)						
	% Organic Matter	Total N (%)	Available P (ppm)	Exchangeable K (ppm)	Cu (ppm)	Mn (ppm)	Mo (ppm)
1-control	3.47	0.18	2566	3412.67	452	443	47.67
2-MYKORICH	3.27	0.18	2985	3082.33	295.67	531	44
3-MYKOVAM	3.47	0.17	3301.67	2957	292.33	477.67	43.67
4-newNFB	3.93	0.23	3857	5171	274	491	44.67
5-newMYC	3.9	0.21	2690	4368.67	335	444.67	6.33
6-combined newNFB+newMYC	3.4	0.19	2630	5922.33	351.67	419	8.33

In the case of Au mined-out soil, the findings reveal that Cu levels are lowest in newNFB-treated seedlings, Mn levels are lowest in the combined newNFB+newMYC-treated seedlings, and Mo levels are low in both newMYC- and combined newNFB+newMYC-treated seedlings. These results emphasize that newMYC and the combined treatment of newNFB+newMYC are the most effective strategies for mitigating heavy metal concentrations in Au mined-out soils.

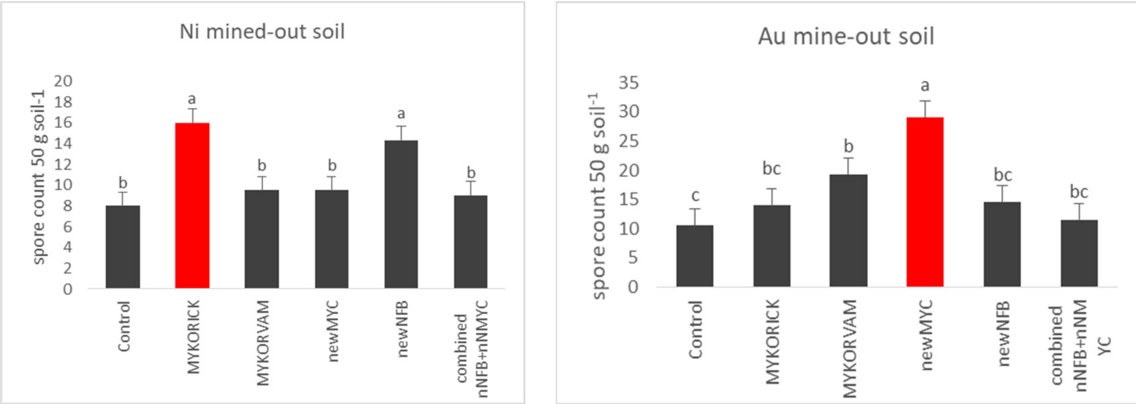
These results demonstrate a notable improvement in the soil status of mined-out areas through the application of microbial biofertilizers and complete fertilizer, leading to enhanced growth in Narra seedlings. Specifically, there is a noteworthy increase in key soil parameters, including nutrient levels and overall soil health. The positive impact is evident in the increased percentages of organic matter, total nitrogen, and available phosphorus. Additionally, a significant reduction in the concentration of heavy metal elements in the soil is observed.

These promising findings highlight the potential of biofertilizers as effective tools for bioremediation, particularly in addressing the challenges posed by degraded soils in mined-out areas. The positive influence on soil quality and plant growth underscores the importance of sustainable and environmentally friendly approaches for soil rehabilitation in mining-affected regions.

3.2. Mycorrhizal Spore Count

The number of spore-forming AMF in the roots of Narra seedlings raised in the two different mined-out soils is shown in Figure 2. One-way ANOVA showed a significant difference ($p < 0.05$) in spore counts between mined-out soils. In the Ni mined-out soil, the spore count in the rhizosphere was highest in the MYKORICH®-inoculated seedlings and lowest in the control (Figure 2a). On the other hand, in the Au mined-out soil, the highest spore count was found in the newMYC-treated seedlings (Figure 2b) and was significantly

($p < 0.05$) different from the other inoculants six (6) months after inoculation, and the lowest was recorded in the control set-up.

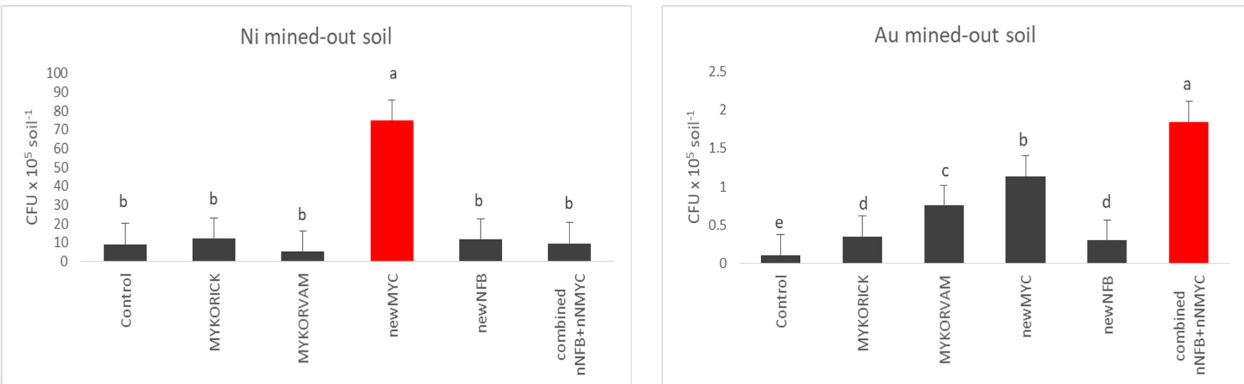


(**a**) (**b**)

Figure 2. AMF spore count in (**a**) Ni mined-out and (**b**) Au mined-out soil (Note: letters above the error bars indicate significance differences at 0.05).

3.3. NFB Colonization

The colony-forming units of NFB in the roots of Narra seedlings grown in the two mined-out soils are shown in Figure 3. One-way ANOVA showed significant results in Narra seedlings grown in Ni mined-out soil ($p < 0.05$). For the Ni mined-out soil, NFB colony-forming units were highest in newMYC-inoculated seedlings and lowest in the MYKOVAM®-treated seedlings (Figure 3a). Moreover, in Au mined-out soil, the highest NFB count was found in the combined newNFB+newMYC-treated seedlings (Figure 3b) and was lowest in the control.



(**a**) (**b**)

Figure 3. NFB colonization in (**a**) Ni mined-out and (**b**) Au mined-out soil (Note: letters above the error bars indicate significance differences at 0.05).

Each treatment's microbial root infection potential was measured through the number of spores in the soil [19]. The mycorrhizal fungi that form symbiotic relationships with the plants are abundant in the soil. From the results, we can glean that the MYKORICH®-inoculated Narra seedlings showed the highest mean spore count in the Ni mined-out soil, while the newMYC-inoculated seedlings provided the highest mean spore count in the Au mined-out soil. Also, Figure 4 below shows the NFB cultures in different treatments of both mined-out soils. It is noticeable that the NFB colony is also present in the control, proving that the mined-out soils of both sites were still evident in their soil, which is a reasonable implication of their soil status. However, their population is still low and cannot sustain the growth of plants in the mined-out area.

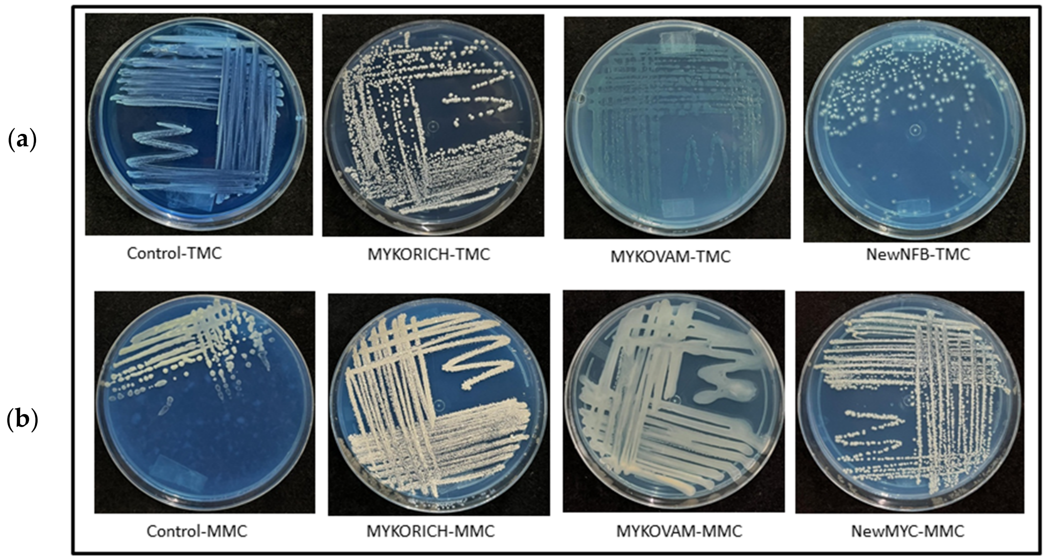


Figure 4. Nitrogen-fixing bacterial cultures on a nitrogen-free Dobereiners agar medium that were extracted from the roots of treated Narra seedlings grown for six months in Ni (TMC) (**a**) and Au (MMC) (**b**) mined-out soils. Photo credit: MYKOVAM Laboratory University of the Philippines Los Baños.

3.4. Microbial Root Colonization

The microbial root colonization exhibited significance differences among treatments ($p < 0.05$). Root colonization was most pronounced in newMYC-treated Narra seedlings compared to the control (Figure 5a) in Ni mined-out soil, and MYKORICH©-treated Narra seedlings were highest in Au mined-out soil (Figure 5b). This result conforms to the study of [22], where the root colonization was higher in plants treated with AMF than in uninoculated seedlings.

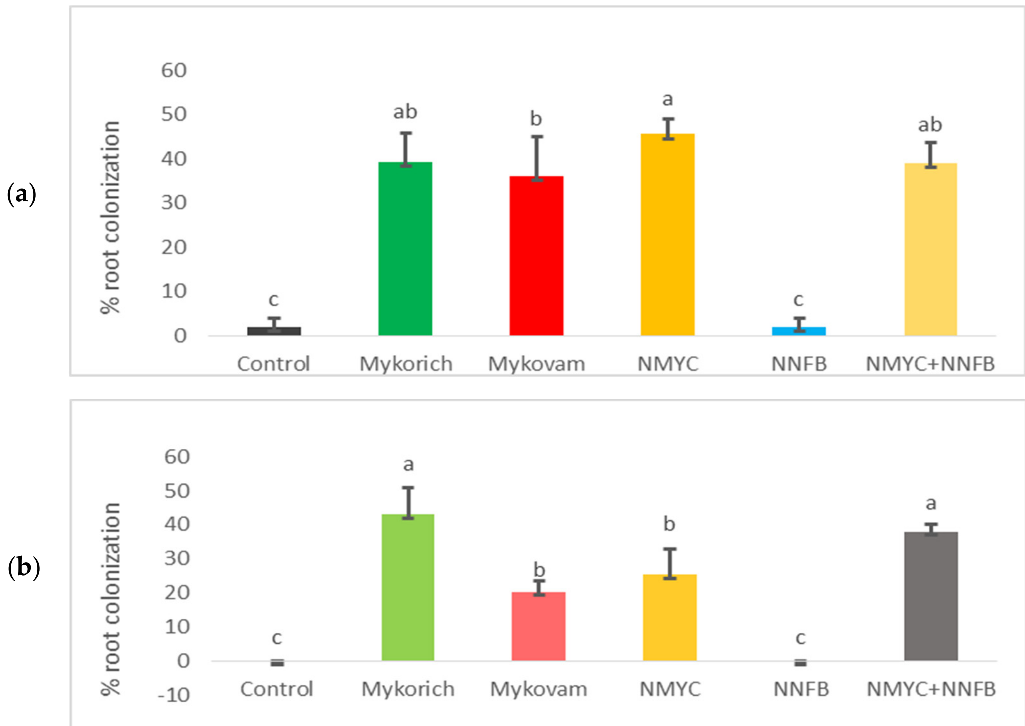


Figure 5. Microbial root colonization (%) in (**a**) Ni mined-out soil and (**b**) Au mined-out soil. (Note: letters above the error bars indicates significance differences at 0.05).

4. Discussion

This study demonstrated that microbial biofertilizers could colonize the mined-out rhizosphere and infect the Narra roots much better than the control. The root colonization results clearly show that MYKORICH® is a good treatment as a soil ameliorator for Narra seedlings grown in mined-out soil. The results further reveal that MYKORICH® was able to produce spores best in Ni mined-out soil, while it also colonizes best in the Au mined-out soil. On the other hand, the newMYC, which were the Marinduque AMF isolates, performed well when exposed to Au mined-out soils. The results showed that newMYC could produce spores best in the Au mined-out soil, the highest NFB colony in Ni mined-out soil, and colonize the root of Narra seedlings raised in Ni mined-out soil. The results demonstrate that the new isolates from the copper mine tailings in Mogpog, Marinduque, thrived best in the Au mined-out soil in the MMC, given their similar microbial population. Since these new isolates from Marinduque are pioneering microbial biofertilizer formulations applied in the soils of MMC, it can be assumed that newMYC can be applied to other Au mined-out areas but this still needs further verification.

In addition, the study of [23] also proved that microbial biofertilizers-inoculated Guar (*Cyamopsis tetragonoloba* Linn.), also a leguminous species, have an enhanced rhizosphere and nutrient uptake of 10–70% maximum compared to the uninoculated control. Moreover, using AMF and NFB as biofertilizers enables plants to use mineral elements such as nitrogen and phosphorous effectively, which significantly improves the plants' nutrition, soil structure, fertility, and heavy metal remediation [24]. Based on the results of this study, MYKORICH®, newMYC, and the combined newMYC+newNFB treatment are the biofertilizers best suited for Ni and Au mined-out soils, respectively, given their promising results in terms of microbial root infection potential.

Mycorrhiza is categorized into two groups, endomycorrhiza and ectomycorrhiza, based on its localization and relationship with the plant roots [25]. Ref. [26] endomycorrhiza is a fungi that colonize the interior of the host plant root cells and form arbuscules and, hence, the term arbuscular mycorrhizal fungi; meanwhile, the fungi in ectomycorrhiza are located outside of the plant root cells. The microbial biofertilizers MYKORICH®, newMYC, and combined newMYC+newNFB are arbuscular mycorrhizal fungi or endomycorrhiza, and the latter is a combination of AMF and nitrogen-fixing bacteria (NFB), respectively. It can be concluded that the AMFs applied in this study provided a very robust association and symbiosis with the Narra seedlings, given the remarkable results of their spore production, NFB colonies, and root colonization. These symbioses can aid Narra seedlings' growth in the mined-out soil assisted by soil amelioration. Ref. [27] explained that AMF symbioses enhanced the uptake of limited nutrients such as phosphorus, zinc, copper, etc., including water from the soil through plant-associated fungal hyphae.

Additionally, AMF form vesicles, arbuscular, and hyphae in roots, spores, and hyphae in the rhizosphere, which significantly enhance the access of the roots to a large soil surface area, causing improvement in plant growth (Bowles et al. 2016) as cited in [28]. As seen in Figure 6 below, vesicles and spores were present in the roots of Narra seedlings six months after inoculation. Moreover, the inoculation of AMF significantly enhances the concentration of various macro-nutrients and micro-nutrients, which leads to increased photosynthate production and, hence, increased biomass accumulation [28–31], which supports the claims on the mechanism behind the phenomenal growth of Narra seedlings raised in Ni and Au mined-out soils inoculated with microbial biofertilizers, specifically with AMF, in addition to compost and the complete fertilizer.

Furthermore, Narra, a leguminous species, is already an NFB-loving plant species that also exhibits an enhanced ability to absorb more nutrients and adapt in heavy metal-contaminated soils when inoculated with NFB-rich microbial biofertilizers. The NFB contributes to the plants' nitrogen requirements through biological nitrogen fixation, an essential biological process for plant growth and development. The symbiotic relationship between the rhizobia and NFB is the formation of nodules (specialized nitrogen structure for nitrogen fixation) on the roots of host legume plants [32], which enable the plants to tolerate the harsh stress conditions of soil salinity, temperature, acidity, alkalinity, drought, and metal toxicities [33]. Nevertheless, the potential interactions between arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing bacteria (NFB) in co-inoculations remain inadequately explored. This gains significance considering the positive impact of mycorrhiza on phosphorus availability, a key factor influencing nodulation by NFBs. Arbuscular mycorrhizal fungi enhance phosphorus uptake in deficient soils by increasing the rate of P uptake per unit of root, attributed to the superior efficiency of hyphal surfaces in comparison to cylindrical root surfaces [34]. And, this uptake of P influences the development of nodules on NFB.

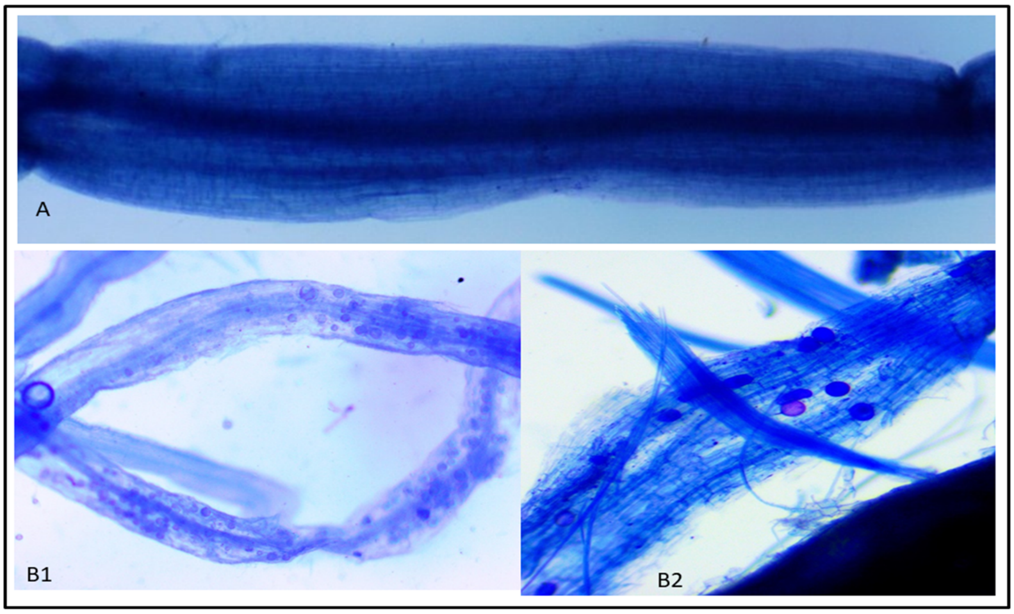


Figure 6. Roots of Narra not colonized (**A**) and those with arbuscular mycorrhizal fungi (**B**) with vesicles (oblong shaped structures in (**B2**)) and spores (round structures in (**B1**) and oblong shaped structure in pink on (**B2**)) six months after inoculation and growth under nursery conditions. Photo credit: MYKOVAM Laboratory University of the Philippines Los Banos.

In addition, the response of the inoculated Narra seedlings raised in metal-contaminated soils was remarkable. A notable mechanism of plants to cope with heavy metal stress is phytochelatins (PCs) induction and synthesis. Berreck and Haselwandter (2001), as cited in [35], explained that PCs are essential peptides synthesized enzymatically from glutathione by phytochelatins synthase in the presence of heavy metal ions. It is well-documented that mycorrhiza could improve plant growth in metal-polluted soils by enhancing metal containment in roots and reducing translocation to the shoots. In addition to AMF's well-known contribution to plant nutrient acquisition and growth, these fungi develop diverse mechanisms that encourage plants to grow in soils with high toxic metal concentrations [27,36,37]. Also, the AMFs can adapt to or resist metal stress, which essentially protects and promotes plant growth, enhancing nutrient acquisition and phytoremediation in heavy metal-contaminated soils [38,39]. Furthermore, detoxification strategies such as avoidance and tolerance have been adopted by plants in response to heavy metal contamination [40,41].

The advanced mechanisms developed in the Narra seedlings treated with different microbial biofertilizers must be considered as one of the rehabilitation strategies for mining companies. The microbial biofertilizers employed in this research, including MYKORICH®, MYKOVAM, newMYC, newNFB, and their combinations, are naturally occurring in the environment and can be easily extracted for bioremediation. Notably, their cost-effective nature offers a distinct advantage, particularly for mining companies seeking efficient solutions for soil amelioration and the rehabilitation of mined-out areas.

5. Conclusions

Rehabilitating mined-out areas has become more evident than ever, particularly with the successful development of a protocol in this study for raising healthy Narra seedlings in a nursery. This achievement contributes to the growing body of research focused on reforestation using specific Narra seedlings. It has been demonstrated that the applied microbial biofertilizers could increase their spores and NFB colonies and colonize the rhizosphere of Narra seedlings raised in mined-out soils.

The major challenge is testing and monitoring how these hardened Narra seedlings can grow and survive in the field. Only then will a working protocol for Narra as a reforestation species be firmly established.

Further, the compost employed for cultivating Narra seedlings could potentially serve as a reservoir of microbes with diverse capabilities. These microorganisms may exert an indirect influence on the observed root colonization and subsequent benefits to the growth of Narra seedlings. Hence, it is recommended to include an experimental control without compost to provide further insights into the role of compost in microbial root colonization.

Additionally, a deeper understanding of the cellular and molecular basis of detoxification with microbial biofertilizer applications such as phytochelatins, metallothioneins, and other related peptides and proteins that protect plants and help them in adapting to metal stress through homeostatic regulation of metals and protection against oxidative stress is further recommended in this study.

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