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Microbial Biofertilizers and Soil Amendments Enhanced Tree Growth and Survival in a Barren Mined-out Area in Marinduque, Philippines

ABSTRACT

A bioremediation protocol was developed for rehabilitating mine tailing areas using microbial biofertilizers and soil amendments. The effects on the growth and survival of tree species in a three-decade barren mined-out area in Barangay Capayang, Mogpog, Marinduque, Philippines were also determined. Three concurrent field experiments were established in June 2016 using Pterocarpus indicus, Acacia mangium and Eucalyptus urophylla. Treatments for this study were uninoculated seedlings and seedlings inoculated with mycorrhiza (MYKORICH® for P. indicus) or Surigao isolate (for A. mangium and E. urophylla) with and without nitrogen-fixing bacteria. Aseptically germinated seedlings were inoculated when they were transplanted from seed boxes into individual polybags. After six months, the seedlings were planted in the mined-out area following Randomized Complete Block Design. The excavated soil were mixed with 1 kg vermicompost and 500 g lime before backfilling the 30 cm^3 planting hole. After one month, 10 g NPK fertilizer and 5 g urea were applied on each seedling. Microbial-inoculated seedlings showed better growth performance with higher plant dry weight and microbial population compared to the uninoculated plants after 27 months. Hence, P. indicus, A. mangium, and E. urophylla inoculated with arbuscular mycorrhizal fungi and applied with lime, vermicompost, and basal inorganic fertilizer could be effective as reforestation species in barren mined-out areas.

Key words: *mine tailing, bioremediation, P. indicus, arbuscular mycorrhizal fungi, nitrogen-fixing bacteria*

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INTRODUCTION

The Philippines is rich in mineral deposits. Fifty operating metallic mines are in the country, including nickel, gold with silver as co-product, copper with gold and silver as co-products, chromite, and iron mines (*MGB 2018*). The mining industry gives major contribution for export that helps the economic sector of the country. However, malpractices during mining operations lead to environmental destruction, such as deforestation, soil erosion, and loss of biodiversity due to mine tailings. Tailings are waste products after milling of ore and extraction of the target minerals.

Mine tailing areas are priority for rehabilitation because the risk impacts are alarming (*Ilagan 2009*). The immediate effects of mining on ecosystems include: destruction of adjacent habitats as a result of sediment runoff from mine sites; pollution due to release of toxic metals and other harmful chemicals by air emissions, acidic mine water and other mine effluents; deposition of dust upon surrounding habitats and creation of potentially unstable landforms (*Doronilla 2012*). With this, increasing accumulation of heavy metals pose hazards to the welfare of the community (*Castillo et al. 2010*).

In 2001, the Mines and Geosciences Bureau (MGB) of the Department of Environment and Natural Resources (DENR) identified seven abandoned mine sites in the Philippines including one mining area operated in Marinduque. A priority list of inactive mining companies in the Philippines was also released by a consulting firm. The list comprised of at least four copper mining companies that include Ino and Capayang Mines that also operated in Mogpog, Marinduque (*Aggangan et al. 2019*). These were open pit mining areas where the mine tailings were dumped in a nearby area where schools, residential areas, agricultural lands, and marine bodies are located.

One strategy to address the threats of mine tailings is through bioremediation technologies. Bioremediation is a process used to treat contaminated media, including water, soil, and subsurface material by altering environmental conditions to stimulate growth of microorganisms and degrade the target pollutants (*Raymundo 2012*). It is the use of plants and their associated microbes to sequester and concentrate heavy metal contaminants (*Raymundo 2005, 2006, Khan 2006*). In many cases, it is less expensive and more sustainable than other remediation alternatives (*Ford and Wilkin 2007*). This technology is relatively new, efficient and cost-effective.

Bioremediation technologies use mycorrhizal fungi which are crucial link between the root system of the plants and the soil. The association is mutually beneficial to the host plant and the fungus (*Smith and Read 2008*). Arbuscular mycorrhizal fungi (AMF) colonize the roots of plants and enhance water and nutrients transport, particularly phosphorous (P), thus, increase growth and yield of crops (*Brundrett et al. 1996, Aggangan et al. 2017, 2019*). Mycorrhizal fungi also improve the soil physical characteristics that help increase particle aggregation and soil stability (*Khan 2006*). Inoculation of these symbionts produce taller, more vigorous, and more drought-tolerant seedlings (*Castillo 1993*). Mycorrhizal fungi, therefore, have indispensable role in the environment especially in stressed conditions like mined-out or mine tailing areas.

The National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB) developed a soil-based biofertilizer containing the spores and propagules of beneficial AMF. Mykovam® is one of the BIOTECH's commercially available biofertilizers, which can be used in almost all plants, such as agricultural crops, fruit and forest trees except pines, dipterocarps, and orchids (*BIOTECH 1995*).

Initial bioremediation studies to rehabilitate copper (Cu) mine tailings in Barangay Capayang, Mogpog, Marinduque used microbial biofertilizers (mycorrhizal inoculants and nitrogen-fixing bacteria developed at BIOTECH-UPLB in combination with soil amendments (lime and vermicompost) (*Aggangan et al. 2017, 2019*).

In the study of *Cadiz et al.* (*2012*) in Capayang mine tailing area, the treatments were lime, and mycorrhizal inoculation (Mykovam® and MineVam from BIOTECH UPLB). Mykovam® consists of five species of mycorrhizal fungi while MineVam contains one species isolated from a grass thriving in a mine tailing area in Paracale, Camarines Norte. The outplanted Jatropha seedlings with no mycorrhizal fungi, vermicompost, and lime exhibited the poorest growth while the tallest and biggest diameter was observed in seedlings treated with Mykovam® or MineVam plus and lime. Plants treated with mycorrhiza plus lime and vermicompost gave the highest leaf, stem, and root dry weights. It was concluded that Jatropha can be grown in an abandoned mine area provided that soil amendments are applied. The best growth of Jatropha was observed when lime, vermicompost, and mycorrhiza were combined. Mycorrhizal treatment increased the root/shoot barrier of Jatropha from heavy metals thus, may help regulate the translocation of Cu, lead (Pb) and zinc (Zn), as well as enhance the heavy metal resistance of the plants.

David et al. (*2012*) evaluated the heavy metals uptake of compost for potential application in treating mine tailings in the Philippines. Metal uptake of soil compost was evaluated through ex-situ experiment where pure soil or soil compost samples were exposed to Ni, Pb, Cd, Al and Cu solutions. Metal uptake generally increased from pure soil to pure soil compost, thus, can be applied to treatment of heavy metals in mine tailings in the Philippines.

Pterocarpus indicus and *Acacia mangium* are both fast-growing evergreen species from the family Fabaceae, commonly known as legumes. These trees are also considered to maintain ecosystem fertility and are used to stabilize soil for revegetation (*Langkamp et al. 1979, Dreyfus and Dommergues 1981*). *Pterocarpus indicus* specifically, is indigenous to the Philippines and is among the best-known trees in Southeast Asia and East Asia extending eastward to the northern and southwest Pacific region (*Thomson 2006*). The genus Pterocarpus is the largest family of flowering plants (*Polhill 1981*). It has abundant lateral roots that increase its root area which is significant for absorption of water and nutrient uptake (*Calora 1992*).

A. mangium distribution area, on the other hand, stretches from Indonesia (i.e., on the islands of Sula, Ceram, Aru, and Iriyan Jaya), Papua New Guinea, and north-eastern Queensland in Australia (*Hedge et al. 2013*). It is widely planted in industrial plantations and for reforestation within degraded lands.

P. indicus and *A. mangium* are fast growing tree legumes well suited for bioremediation due to their rapid growth and tolerance for very acidic and infertile soil. As a nitrogen fixer, both are also well adapted to low available water conditions and nutrient (*Gazal et al. 2004*). On the other hand, *Eucalyptus urophylla* is under the large family of Myrtaceae. This tree has distinct features which include fast-growth, high cellulose production with low susceptibility to diseases and uncontrolled conditions (*Santos et al. 2001*). They are also considered important for their role on wood production in Asia (*Midgley and Pinyopusarerk 1996*).

The experiment in Barangay Capayang, Mogpog, Marinduque mine tailing area established in June 2016 was the first field trial to study AMF- *P. indicus*, AMF- *A. mangium*, AMF- *E. urophylla* symbioses. Innovations from the protocol used by *Aggangan et al.* (*2017*) on Jatropha were: increasing the amount of lime and vermicompost; and adding complete fertilizer (14-14-14 NPK) and urea. Thus, the aims of the present study were to develop bioremediation protocols for rehabilitating mined-out and mine tailings areas using microbial biofertilizers (mycorrhizal inoculants and NFB developed in the Philippines) and soil amendments (lime and vermicompost), determine the effects of microbial biofertilizers and soil amendments on the survival and growth of different tree species in a three-decade barren mined-out area in Barangay Capayang, Mogpog, Marinduque; and determine the effects of mycorrhizal inoculation on rhizosphere microbes of *P. indicus*, *A. mangium* and *E. urophylla* planted in a three-decade barren abandoned mined-out area.

MATERIALS AND METHOD

Study Site

The field site is a two-hectare area geographically located in Barangay Capayang, Mogpog, Marinduque (N13029'54" and S121052'12"E) (**Figure 1**). It is part of a 32-hectare open pit mined-out dumpsite that has been unattended since 1996 after extracting copper (Cu), the residual heavy metal after gold extraction from ore. The research area is a plateau on top of a hill at 60 m above sea level. It is overlooking the Mogpog Elementary School and the Capayang National Comprehensive High School surrounded by various ecosystems (e.g., mangrove, agricultural, river and marine) and communities. Their location is at risk because of the instability of the abandoned mine dumpsite. The site is predominantly barren with patches of *Acacia auriculiformis, Saccharum spontaneum, Pityrogram macalomelanos, Muntingia calabura* and *Trema orientalis* and different species of ferns.

The soil contained Cu, lead, cadmium, and zinc but only Cu exceeded the maximum allowable limit. It was almost double than that of the allowable limit of 36 mg kg^{-1} of soil. Cu is one of the waste heavy metals during gold extraction in the mining process. The soil is acidic (pH 4.6±0.25, 1:1 soil water) with low organic matter content (0.34±0.14%), Total N (0.22±0.02%), available Bray P (75.5±62 ppm), and low in exchangeable K (0.12±0.08 me/100 g soil), Ca (6.78±2.45 me/100 g soil), Mg (3.48±1.13 me/100 g soil) and CEC is 18.53±3.52 me/100 g soil. It contained Cu (100 mg kg^{-1} soil), Pb, Cd, Fe and Zn.

The impact of the soil degradation poses environmental hazards to the community and to all other living biota within the vicinity. Hence, it is a priority site for rehabilitation to prevent leaching of heavy metals to the river system, agricultural areas, mangrove and marine ecosystems.

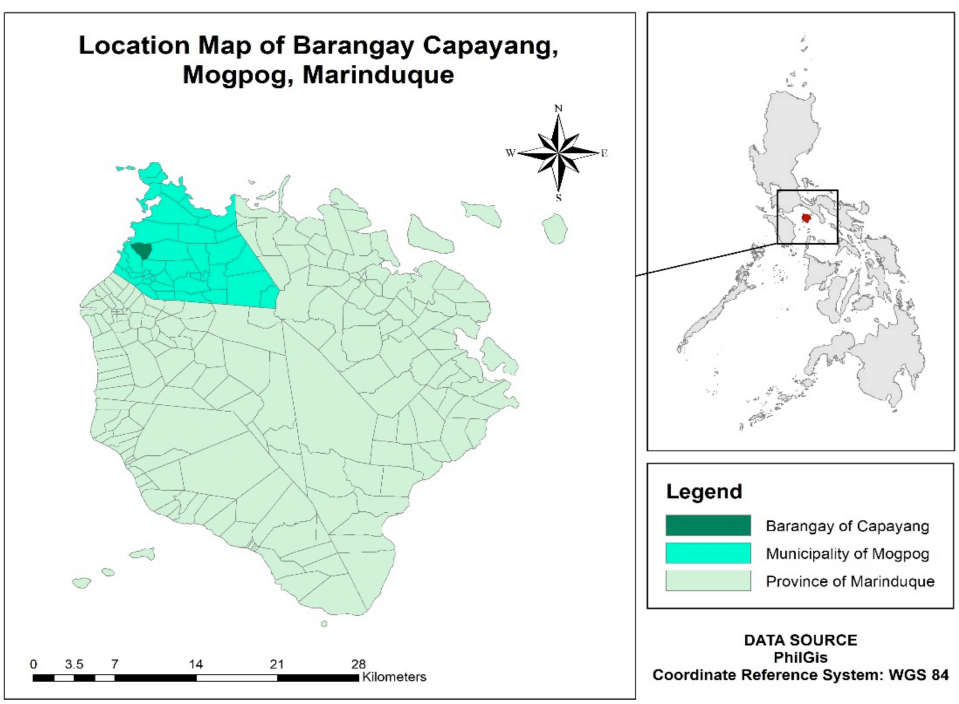

Figure 1. Location Map of Barangay Capayang, Mogpog, Marinduque, Philippines generated in ArcMap 10.3.1 software.

Experimental Design

Three field experiments were established following a Randomized Complete Block Design (RCBD) with a spacing of 2 m x 2 m. For *P. indicus*, the experiment consists of five blocks with ten seedlings in a row per treatment while for *A. mangium* and *E. urophylla*, the experiment (each tree species) had four blocks and five seedlings per row per treatment (**Table 1a**). For *P. indicus*, three mycorrhizal inoculants (MYKOVAM®, MYKOCAP® and MYKORICH®) were studied; however, 27 months after field planting, tree representatives from three inoculation treatments were excavated to assess the different parameters observed in this experiment (**Table 1b**).

Table 1a. Inoculation treatments per tree species 12 months after field planting.

P. indicus	*A. mangium*	*E. urophylla*
Control	Control	Control
BioN*	BioN*	BioN*
MYKOVAM®	*Glomus macrocarpum*	*Glomus macrocarpum*
MYKOCAP®	Surigao isolate**	
MYKORICH®	BioN	Surigao isolate**
MYKOVAM®+BioN	Gmacrocarpum+ BioN	
MYKOCAP®+BioN	Surigao+BioN	
MYKORICH®+BioN		Surigao+BioN

*NFB in BioN contains Azospirillum spp., a nitrogen-fixing bacteria.

**Surigao isolate originally came from a mine tailing in Placer, Surigao, Mindanao.

MYKOVAM® is a soil-based mycorrhizal inoculant containing 12 species of AMF while MYKOCAP® is similar to MYKOVAM® but in capsule form for easier application. MYKORICH® is a sand-based mycorrhizal inoculant containing 12 species of AMF similar to that of MYKOVAM® and MYKOCAP®, which was formulated to address export demand. It is in capsule and powder form for easier application.

Table 1b. Inoculation treatments per tree species excavated or samples at 27 months after field planting.

P. indicus	*A. mangium*	*E. urophylla*
Control	Control	Control
MYKORICH®	Surigao isolate**	Surigao isolate**
MYKORICH®+BioN	Surigao isolate+ BioN	Surigao isolate+ BioN

Preparation of Planting Materials

Seeds of *P. indicus* were collected from phenotypically and genetically superior plus trees growing in the vicinity of the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB). With the recommendation of the Department of Environment and Natural Resources (DENR)- Boac, Marinduque, the seeds were given to an accredited nursery operator in Gasan, Marinduque where the seedlings were raised from February 2016 to May 2016.

Seeds of *A. mangium* and *E. urophylla* were obtained from the Ecosystems Research and Development Bureau, DENR, Los Baños, Laguna, Philippines which, according to the Bureau, were collected from selected plus trees seed orchards in Mindoro, Philippines. Seedlings were raised at BIOTECH-UPLB and transported to Marinduque prior to field planting in June 2016.

Inoculation with Microbial Biofertilizers

P. indicus seedlings were inoculated with mycorrhizal inoculants (MYKOVAM®, MYKOCAP® or MYKORICH®) with and without BioN (containing NFB belonging to the genus Azospirillum isolated from the roots of *Saccharum spontaneum* (**Figure 2**). Each seedling was inoculated with either 5 g of MYKOVAM® or two capsules of MYKOCAP® or MYKORICH® placed in two holes 2 cm away from the base of the stem. Inoculation was done one month after pricking. BioN was inoculated into seedlings placed in a hole at 5g per seedling.

For *A. mangium* and *E. urophylla*, seedlings were either uninoculated or inoculated with AMF *Glomus macrocarpum* or Surigao isolate singly or in combination with BioN (in green packaging) containing NFB. Surigao

Figure 2. Microbial biofertilizers developed at BIOTECH-UPLB that were used in this study.

isolate is an AMF isolated from the mined-out area of the Manila Mining Corporation in Placer, Surigao, Mindanao, Philippines. This isolate was the most promising AMF from that mined-out site the authors observed 12 years ago. Each seedling was inoculated with 100 spores of either *G. macrocarpum* or Surigao isolate with and without 5 g BioN inoculant containing 1 x 10^8 CFU of NFB.

Inoculation was done during transfer of seedlings from germination boxes into individual polybags filled with oven-sterilized soil sand (1:1 v/v) mixture. This medium was found favorable for growth of microbes that will eventually help the plant grow better. Microbial biofertilizers were placed beneath the roots of seedlings in order that the microbes will immediately infect the roots and improve early growth and survival of plants.

Establishment of field experiments

In June 2016, seedlings were planted in a mine tailing in Barangay Capayang, Mogpog, Marinduque. The seedlings were planted in the field with the following protocols: Each seedling was planted in 30 cm^3 hole and applied with 500 g lime and 1 kg vermicompost; Vermicompost and lime were mixed with the excavated mine tailing soil before backfilling the planting hole; Five grams of complete fertilizer (NPK), 5 g urea plus 500 g vermicompost were applied per seedling one month after field planting.

Vermicompost is the final product of a process called vermicomposting which uses earthworms and microorganisms to naturally break down organic materials. According to *Chaoui et al.* (*2003*), vermicompost has been proven to be an enhancer of plant growth. It is also observed to enhance the microbial activity of the soil and increase oxygen availability, soil porosity, and water infiltration (*Arora et al. 2011*). The vermicompost used in this study was obtained from Lipa City, Batangas, Philippines. It has a 36% organic matter concentration, 15:1 CN Ratio, 1.89% nitrogen, 2.49% phosphorus (P_2O_5), 1.40% potassium (K_2O), and other micronutrients.

The control seedlings were planted by the members of the Marinduque Council for Environmental Concern (coded in the graphs as MACEC) adjacent to the experiments of this study. No soil amendments were applied to the control seedlings. The experiment served as the negative control for the experiment on *P. indicus*. A written consent was received from MACEC for their experiment to be used as demo trials in this study.

Parameters gathered

The height and stem diameter of the experimental plants were measured and recorded. The number of seedlings that survived was recorded. Data recording was done once every three months up to 27 months after field planting. At 27 months, three representative trees from each treatment were excavated to determine growth, total plant biomass, and microbial population in the rhizosphere (mycorrhizal spore count, culturable fungi and NFB) and in the roots (mycorrhizal root colonization). Leaf, branch, stem, and root samples were also taken from the excavated tree samples. These were brought to BIOTECH-UPLB where the partitioned plant samples were cleaned under running water and then air-dried, wrapped separately with paper towel, placed inside brown paper bags and oven-dried at 60°C for five days. Dry weights were measured using an analytical weighing balance.

The population of NFB in the rhizosphere soil was determined using a nitrogen-free malate medium (*Estrada-De Los Santos et al. 2001*) where 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 $MgSO_4.7H_2O$, 0.1 g $MnSO_4.H_2O$, 0.9 g K_2HPO_4, 10 mg $FeSO_4.7H_2O$, 5.0 mg $Na_2MoO_4.2H_2O$, 3.0 mL Bromothymol blue, 0.5% alcohol solution, and 15.0 g agar. All components were dissolved in 1 L distilled water and the pH was adjusted to 7.0 using 1 M NaOH.

For the assessment of mycorrhizal root colonization, 0.2 g $plant^{-1}$ fine roots (< 0.2 mm diameter) were cleared with 10% KOH in water bath set at 90°C for 30 min. It was cooled at room temperature and rinsed with 50% H_2O_2 until the roots became colorless. The H_2O_2 was replaced with 0.1 N HCl, washed in water and then stained in 0.05% methylene blue with 70% glycerine for 30 min in 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 *Brundrett et al.* (*1996*) was used to determine root colonization.

Statistical analyses

All the data were subjected to Analysis of Variance (ANOVA) of a Randomized Complete Block Design (RCBD). Treatment means were compared using Tukey's test at $p<0.05$. Statistical analyses were done using the MSTATC computer program of Michigan State University (*MSU 1989*).

RESULTS AND DISCUSSION

Plant growth

After one year, *P. indicus* inoculated with microbial biofertilizers showed a survival of 98%, *A. mangium* of 95% and *E. urophylla* of 97%. At 27 months, the survival rate of the plants with microbial biofertilizers plus lime, vermicompost, and NPK was 92-95% while the survival rate of the plants in soil without any amendments (planted by MACEC) was 15% only. The inoculated *P. indicus* seedlings exhibited 34.75 cm average height, which is five times higher than the average height (7.40 cm) of the seedlings in soil without any amendments (control experiment managed by MACEC) six months after field planting (**Figure 3A**).

After another six months, the average height of the inoculated *P. indicus* seedlings was 78 cm. It grew taller than the control counterpart (36 cm). Stem diameter doubled with the application of soil amendments. The survival rate of plants with microbes, lime and vermicompost was 95% compared to the 26% survival rate of the uninoculated plants.

BioN and *G. macrocarpum* applied singly on *A. mangium* gave comparable height but significantly taller than the control. After 15 months, the mycorrhiza-inoculated *A. mangium* grew more than a meter in height with healthy green leaves. In contrast, the control *A. mangium* grew poorly with yellow leaves. There seem to be a synergy between *G. macrocarpum* and BioN for *A. mangium* but no clear effect on height and stem diameter (**Figure 3B**). On the other hand, stem diameter of *E. urophylla* increased by almost two times when inoculated with *G. macrocarpum* alone (**Figure 3C**). Stem diameter was reduced when co-inoculated with BioN. In general, inoculation with mycorrhiza alone (MYKORICH® or Surigao isolate) gave the heaviest total plant dry weight and root dry weight of *P. indicus, A. mangium* and *E. urophylla* (**Figure 4**).

Microbial population

The highest culturable fungi was obtained in MYKORICH inoculated *P. indicus* and in the combined Surigao+BioN inoculated *A. mangium*, 27 months after field planting (**Figure 5**). The growing medium was oven-sterilized; however, the rhizosphere soil population of culturable fungi was still highest in MYKORICH®-inoculated seedlings. The lowest was observed in the uninoculated (no microbes) plants. On the other hand, the nitrogen-fixing bacterial count was highest in plants inoculated with BioN (containing nitrogen-fixing bacteria of the genus Azospirillum) but it was comparable with that obtained in MYKORICH®, MYKORICH®+BioN or the control seedlings (**Figure 6**). The MYKORICH® treatment gave the highest percentage of root colonization and spore count (**Figure 7**) that is also significantly different from among all other treatments 27 months after field planting. Treatments with Surigao and Surigao+BioN in *A. mangium* are comparable with each

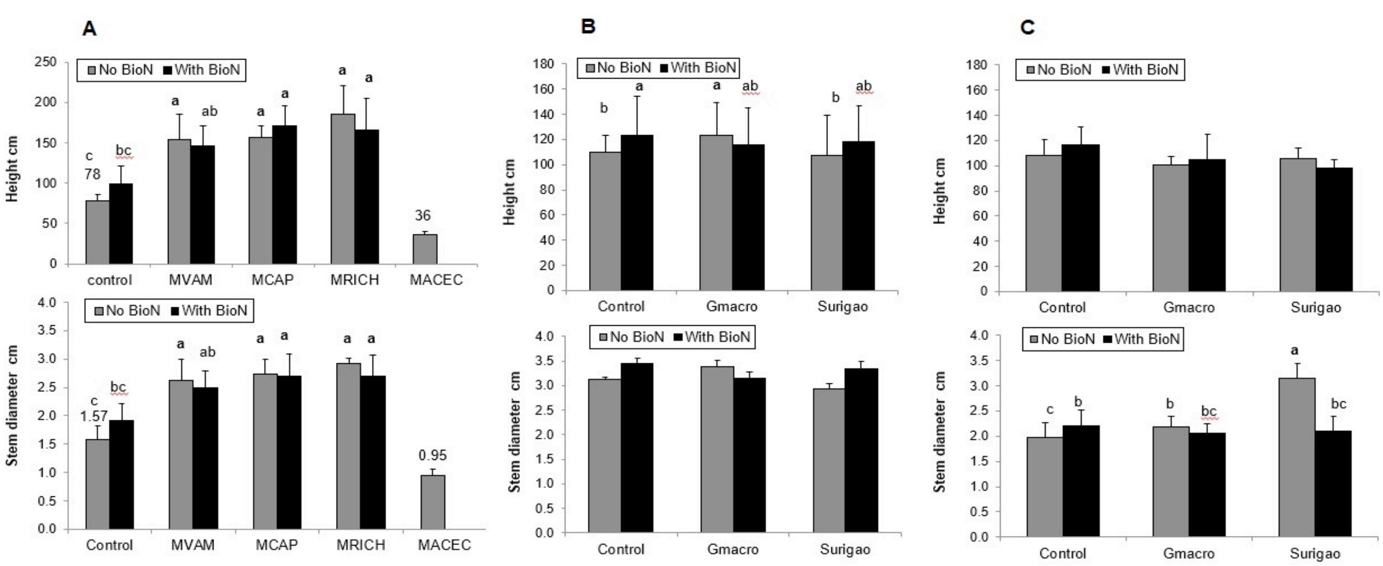

Figure 3. Height and stem diameter of *P. indicus* (A), *A. mangium* (B), and *E. urophylla* (C) seedlings with and without BioN, 12 months after field planting in a mined-out area in Barangay Capayang, Mogpog, Marinduque due to inoculation with mycorrhizal fungi with and without BioN (NFB). Bars with the same letters are not significant from each other using Tukeys test at p<0.05. N=50 for *P. indicus*, N=20 for *A. mangium* and *E. urophylla*.

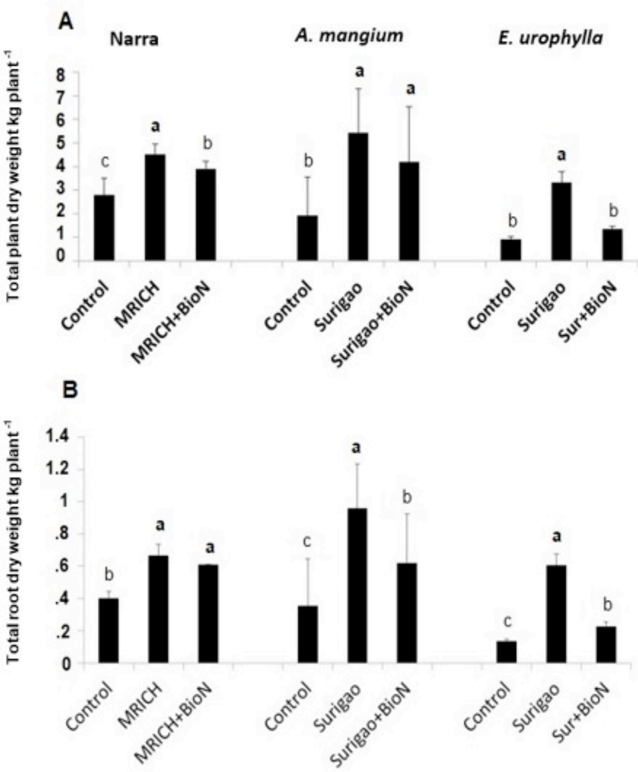

Figure 4. Total plant (A) and root (B) dry weight of *P. indicus*, *A. mangium* and *E. urophylla* 27 months after field planting in a mined-out area in Barangay Capayang, Mogpog, Marinduque due to inoculation with mycorrhizal fungi with and without BioN (NFB). Bars with the same letters are not significant from each other using Tukeys test at p<0.05. n=3.

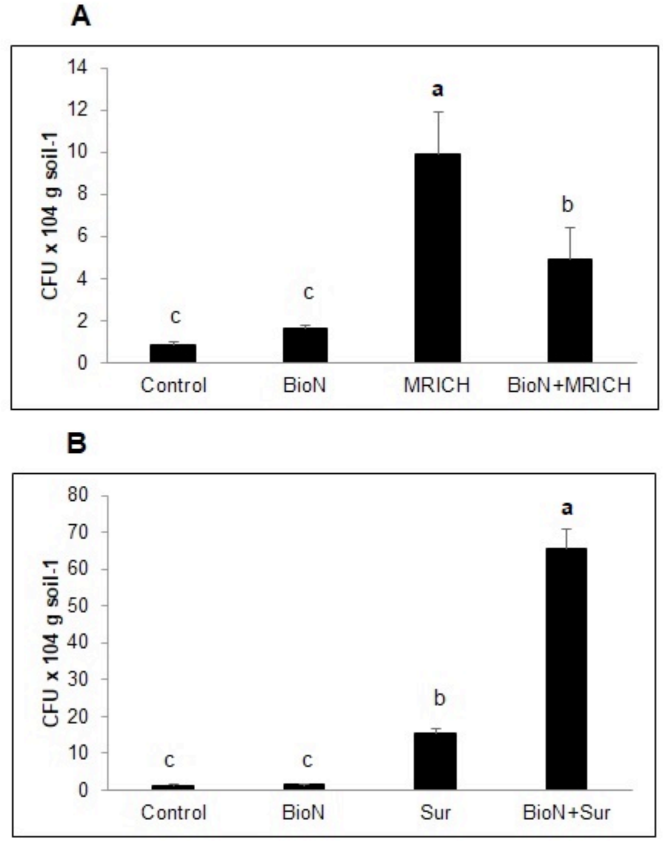

Figure 5. Culturable fungi in the rhizosphere of P. *indicus* (A) and *A. mangium* (B), 27 months after field planting in a mined-out area in Barangay Capayang, Mogpog, Marinduque due to inoculation with mycorrhizal fungi with and without BioN (NFB). Bars with the same letters are not significant from each other using Tukeys test at p<0.05. n=3.

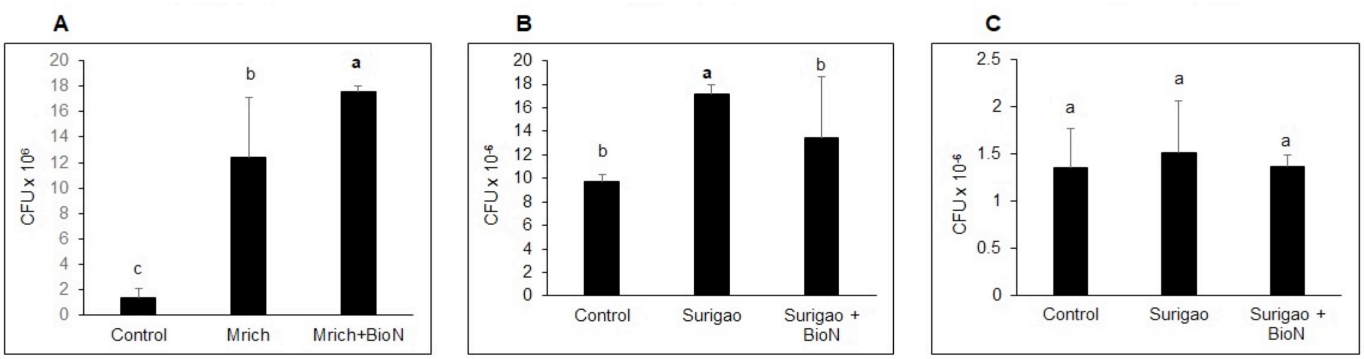

Figure 6. Culturable nitrogen-fixing bacteria in the rhizosphere soil of *P. indicus* (A), *A. mangium* (B), and *E. urophylla* (C), 27 months after field planting in a mined-out area in Barangay Capayang, Mogpog, Marinduque due to inoculation with mycorrhizal fungi with and without BioN (NFB). Bars with the same letters are not significant from each other using Tukeys test at p<0.05. n=3.

other, same with the control on both *P. indicus* and *A. mangium*.

There is a great hope to put back green vegetative cover on the three-decade barren mined-out areas. In the project site, there was no significant temporal changes from 2006 to 2016 (**Figure 8**). This barren state of the mined-out experimental site (encircled area), turned into a mini forest with green vegetative cover in 2018 as a result of the bioremediation study (**Figure 9**).

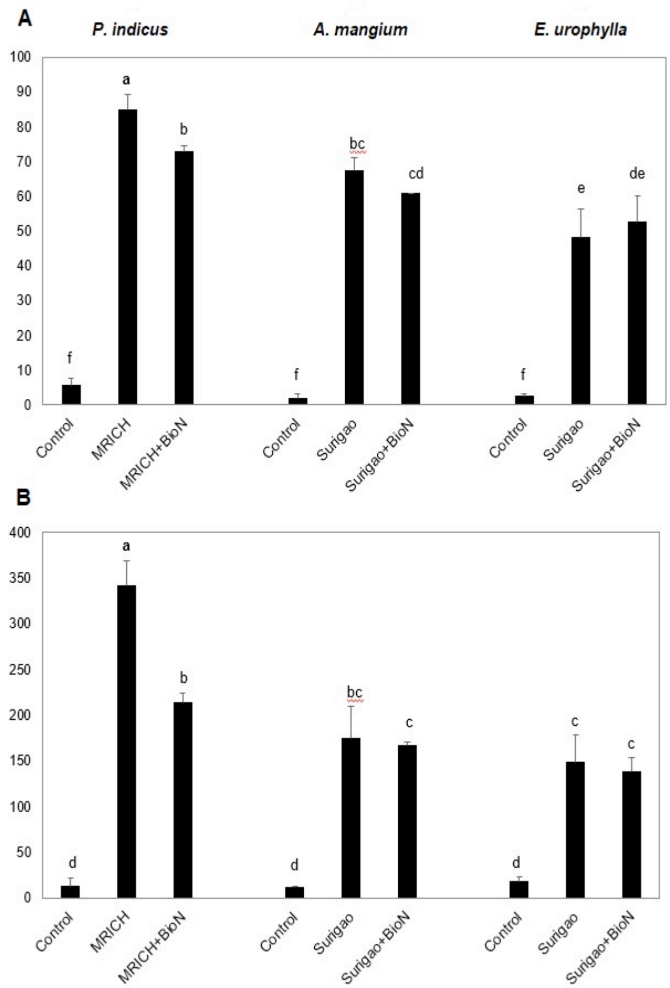

Figure 7. Percent mycorrhizal root colonization (A) and spore count per 50 g soil (B) on *P. indicus, A. mangium*, and *E. urophylla*, 27 months after field planting in a mined out area in Barangay Capayang, Mogpog, Marinduque. Bars with the same letters are not significant from each other using Tukeys test at p<0.05. n=3.

The growth and microbial population of the *P. indicus, A. mangium* and *E. urophylla* seedlings were greatly improved by the microbial biofertilizers and the applied soil amendments. Without these applications, plant growth was stunted with very low survival rate (15%) as compared to 92-95%. The inoculated seedlings grown in soil with lime and vermicompost grew taller and better compared to the uninoculated seedlings grown in soil without microbes and soil amendment.

The pH level of the mined-out site was 3.4-4 which was very acidic and made the area unproductive for more than three decades. However, the application of lime and vermicompost raised the pH level which made the soil favourable to soil microbes that enhanced plant growth and survival. In all the experiment for this study (except for the control under the MACEC experiment), vermicompost and lime were added which showed better growth and survival rate.

Mycorrhizal fungi applied in combination with NFB could promote better plant growth as the plants' requirements for nitrogen and phosphorus would be made available. In this study, there was no synergistic effect of the combined inoculation treatment. The combined inoculation of NFB and AMF, whether MYKORICH® or Surigao isolate, gave lower values than when inoculated with AMF alone in all parameters observed except the parameter on the culturable fungi on *A. mangium* and culturable NFB on *P. indicus*. The combined application did not show superior results. This indicates that it is not practical to use BioN because it does not result to significant improvement of growth when combined with mycorrhiza. Mycorrhiza alone is sufficient. Studies have been made to explore the effect of AMF-fungi on free-living nitrogen fixers in the rhizosphere of legumes (*Lok 2011, Javaid 2010*). These fungal associations are critical during and after soil disturbance because of their role in the establishment and survival of plants (*Miller and Jastrow 1992, Haselwandter and Dowen 1996*). From these experiments, the seedlings with dual inoculation were better than the uninoculated ones compared to single application treatment or control.

Several studies have revealed that mycorrhizal fungi can form a symbiosis with AMF-*P. indicus* (*Castillo 1993, Aggangan and Cortes 2018, Lok 2011, Giri et al. 2018*), AMF-*A. mangium* (*Aggangan et al. 2009, Ghosh and Verma 2006, Satter et al. 2007, Jeyanny et al. 2011*) and AMF- *E. urophylla* (*Adjoud 1996, Chen et al. 1998, Chen et al. 2000*). These researches reported increased plant growth, resistance, and build-up of microbial population in the rhizosphere, particularly under adverse environmental conditions specifically on the stabilization of heavy metal soils. In this study, MYKORICH® and Surigao mycorrhizal isolate promoted the highest microbial population while the lowest were the control or uninoculated seedlings.

Moreover, Surigao isolate promoted higher plant and root dry weights on *A. mangium* and *E. urophylla* than in *P. indicus* applied with MYKORICH® that contains different genera. *Moreira et al.* (*2006*) discussed that these variabilities are mostly influenced by factors inherent to the host plant, climatic and edaphic factors, effects of the soil community, and by the interactions of all factors. It was also observed that there is a direct relationship between root colonization and spore count. However, sporulation rate is very dynamic within its same sampling

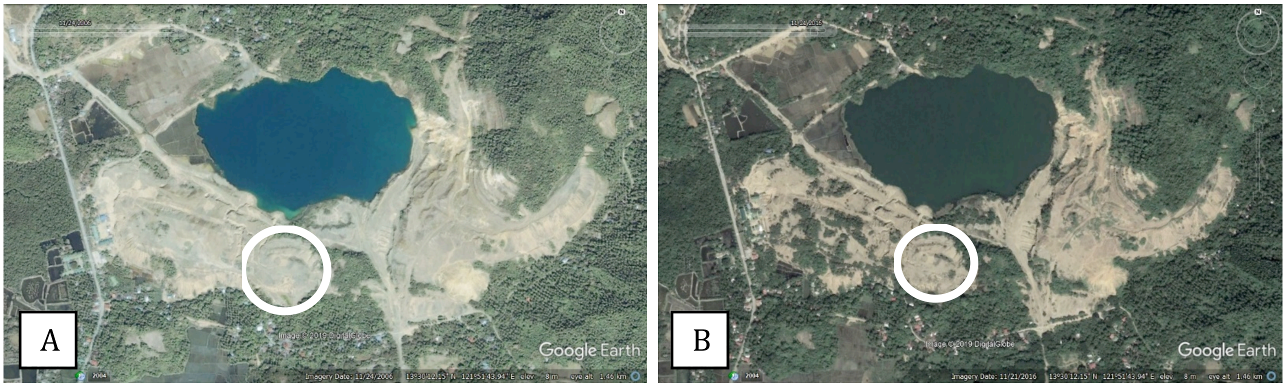

Figure 8. Comparative appearance of the open pit mining site in Capayang, Mogpog, Marinduque from 2006 (A) to 2016 (B). Encircled area is the field site.

Figure 9. Transformation of the barren mined-out site from newly planted (A) to 18 months after field planting with *P. indicus* (B); and 27 months after field planting with *A. mangium* and *E. urophylla* (C).

point in relation to different seasons of the year. This implies then, that the number of spores is not always a reliable parameter to determine the composition of the mycorrhizal fungi community in an ecosystem because some fungi sporulate more, while others do not. This parameter however, is very important in selecting a candidate mycorrhizal inoculant: that is, choose those with high spore count and promote the best plant growth in a short period of time and those that persist in the field with time. The chosen microbial biofertilizers should survive as long as the associated hosts are alive for a more sustainable ecosystem, higher plant yield coupled with improved soil nutrient status.

Since Eucalyptus is also one of the most widely used species for commercial plantation, it is generally monospecific and has been managed successfully and sustainably for many years (*Forrester et al. 2006*). However, mixed-species plantations rise and gain popularity within Eucalyptus and nitrogen-fixing species because they have the capacity to improve soil fertility, nutrient cycling, biomass production, and carbon sequestration. These nitrogen-fixing trees are known to have symbiotic relationship with AMF that is important for forest restoration on degraded lands (*Lok 2011, Bento et al. 2012*). Biological nitrogen fixation (BNF) is discovered by Beijerinck in 1901 (*Beijerinck 1901 in Wagner 2011*). The converted ammonia (NH_3) will then be used by the plants as fertilizers. In this case, BioN containing *Azospirillum* bacteria comprises plant-growth-promoting bacteria (PGPB) and can fix atmospheric nitrogen (*Fukami et al. 2018*). Several studies also indicate that this interaction may lead to the enhancement of bioremediation in plants (*Xun et al. 2015, Lingua et al. 2013*).

CONCLUSION AND RECOMMENDATION

Mycorrhizal inoculation and application of lime vermicompost, and NPK fertilizer during early seedling stage of *P. indicus, A. mangium* and *E. urophylla* gave significantly higher seedling survival and growth after 12 and 27 months of field planting in a mine tailing area in Capayang, Mogpog, Marinduque, Philippines. The seedlings previously planted in the mined-out area showed very low survival rate, stunted growth, and unhealthy seedlings as indicated by the yellow leaves of the plants. With the application of the mycorrhiza-vermicompost-lime-NPK protocol, the seedlings planted gave higher survival rate, healthy seedlings, and good growth.

P. indicus, A. mangium, and *E. urophylla* inoculated with mycorrhizal biofertilizers and applied with lime, vermicompost, and small amount of basal inorganic fertilizers can be valuable for the reforestation of degraded lands. It has been proven that within two years, the previously barren area for three decades is now exhibiting a young mini forest cover. Therefore, it can be deduced that the technology can be used in the rehabilitation of the mined-out area, including other mined-out areas with the same condition, not only in Mogpog, Marinduque but in other mine tailing areas in the country.

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