



Screening mined-out indigenous mycorrhizal fungi for the rehabilitation of mine tailing areas in the Philippines

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Abstract

Arbuscular mycorrhizal fungi (AMF) play a significant role for mine tailing rehabilitation due to their sensitivity towards a range of soil pollutants. This beneficial biological agent can enhance plant tolerance to heavy metal contamination. This study screened indigenous AMF associated with growing indigenous ferns and grasses in the mine tailings for potential use in rehabilitating a 3-decade abandoned mined out area in Mogpog, Marinduque. *Pterocarpus indicus* Willd. (narra) was used as the host plant to establish mycorrhizal fungi association. Among the treatments, indigenous AMF associated with Ferns 1, 2 and 5 generally improved the height and shoot diameter of the narra seedlings and the effect was comparable with commercially available AMF inoculants, MYKOVAM[®] and MYKORICH[®]. The dry weight of the roots and nodules was consistently improved by indigenous AMF from Fern2 and Grass1, which had comparable effect with MYKOVAM[®] and MYKORICH[®]. Overall, the total seedling dry matter of narra seedlings was significantly stimulated by AMF, irrespective of isolates' origin. The mycorrhizal root infection by AMF and number of spores in the soil were all high as compared with the uninoculated control counterpart. Lastly, AMF inoculation induced Cu retention in the roots of the seedlings. Thus the results imply that, the mined out indigenous AMF are potential agents to rehabilitate the abandoned mine tailings in the Philippines.

Keywords

Ferns; Grasses; Mycorrhizal inoculants; MYKORICH[®]; Narra; *Pterocarpus indicus*

Contents

1	Introduction	72
2	Materials and methods	73
3	Results	75
4	Discussion	81
5	Conclusion	82
6	Acknowledgement	82
7	References	83

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1 Introduction

Bioremediation is a waste management process that potentially neutralize or remove pollutants from a contaminated site (Ritter and Scarborough 1995). Bioremediation strategies have been useful in detoxifying soil contaminants (e.g. heavy metals) by mainly using specific agents such as plants, bacteria, fungi, and algae, which are either indigenous or extraneous in the affected environment (Biswas et al. 2015). Immobilization of heavy metals through bioremediation can be useful in rehabilitating contaminated soils (Wuana and Okieimen 2011; Mahmoud and El-Kader 2015). This strategy is promising because it uses naturally occurring microorganisms and can alleviate various waste products, which are necessary to reduce the metal-contamination associated risks, make the land resource available for agricultural production, enhance food security, and scale down land tenure problems (Wuana and Okieimen 2011).

Excessive heavy metal contamination in mined out areas is a major environmental issue (Bleeker et al. 2002). Abandoned mined out areas are primarily problematic especially to the neighboring communities due to the dumped mine tailings. The tailings are harmful to indigenous plants and animal species and even human life once they reached the water supply system (Bleeker et al. 2002).

In the Philippines, mining operations are prominent, and many are still actively extracting natural products but due to the unregulated practice and improper disposal of mine tailings, it potentially puts the local community and environment at risk. According to National Integrated Protected Areas System (NIPAS), there are 50 active metallic mines in the Philippines that are surely accompanied with mine tailings and mined-out areas and 300 ha of these areas are considered abandoned. Example is gold and copper mine by the Consolidated Mines Inc. in Barangay Capayang, Mogpog, Marinduque, which has been abandoned for more than 30 years. This 3-decade abandoned mined out area has contributed significant consequences to human health especially to the neighboring communities and surrounding ecosystems (Car et al. 2003). There is an urgent need to establish vegetation, in order to reduce the risk of diseases (e.g. cancer, asthma, upper respiratory disorder, etc.) triggered by heavy metal-contaminated water, food, and air.

Nowadays, several microorganisms were successfully used in bioremediation of heavy metal-polluted soils including arbuscular mycorrhizal fungi (AMF). AMF are naturally occurring biotrophs that exchange mutual benefits with about 80% of terrestrial plants (Berruti et al. 2016). AMF confers plant tolerance to heavy metals (Smith and Read 2010) and help alleviate metal toxicity to plants by reducing the translocation of these heavy metals from root to shoot (Leyval et al. 1997; Aggangan et al. 2017). Several studies demonstrated the potential benefits of AMF in ameliorating the heavy metal accumulation to plants through AMF-plant association (Davies et al. 2001; Wang et al. 2007; Karimi et al. 2011; Yang et al. 2015; Jiang et al. 2016). Hence, AMF play a significant role in the success of rehabilitation of stressed environmental conditions such as the mine tailings and mined out areas. In the study site, ferns in the gullies and few grasses on the plateau area were observed. Thus, it was thought that, the associated microbes may facilitate the rehabilitation and transformation of these areas, to bring back green cover and dead soil to life.

The indigenous legumes tree species have a priority in the Philippines reforestation programs. Example is narra (*Pterocarpus indicus* Willd.) of the Family

Fabaceae. Narra is classified as one of the premium tree species because of its high quality wood, which is excellent in making furniture and walling in houses. It bears bright yellow flowers that make it a good candidate in landscaping. Narra is the Philippine national tree, but is now considered as a critically endangered native tree species. Thus, it has been given priority concerns for conservation and reforestation due to its economic, industrial, and ecological value (Delos Reyes et al. 2016). Narra is one of the most valuable native reforestation species, which is found to contain medically important substances (Khan and Omoloso 2003; Ragasa et al. 2005; Cha et al. 2016) but there is little information of their use as host plant in bioremediation.

As a legume, the narra roots have nodules formed by nitrogen-fixing bacteria (NFB) and likewise associated with mycorrhizal fungi, which are phosphorus fixing microbes. Narra can produce new roots in response to reduced soil moisture, which enables them to counteract transplanting shock ensuring survival (Gazal et al. 2004). These characteristics make them potential to revive a 3-decade abandoned mined out areas through the help of AMF association. Narra can grow very well in good soil as well as in a much stressed environment especially when inoculated with NFB and mycorrhizal fungi.

To provide an alternative way of rehabilitating the mined-out areas, this study: 1) screened indigenous mycorrhizal fungi associated with ferns and grasses for potential use in bioremediation of a 3-decade abandoned mine tailings, 2) determined their potential as growth promoter of narra seedlings grown first in soil sand mixture and then transferred in soil from mine tailing area in Mogpog, Marinduque, and 3) compared the performance of the mine tailing mycorrhizal isolates, with the commercial mycorrhizal inoculants MYKOVAM® and MYKORICH®, in terms of their potential as plant growth promoter.

2 Materials and methods

Description of the mine tailing area: The two hectare study site is geographically (N13°29'54" and S121°52'12"E) located in Barangay Capayang, Mogpog, Marinduque. It is part of the 32 ha open pit mined out dumpsite that has been unattended since 1996 after extracting Cu from ore, which is the residual heavy metal after gold extraction.

The mine tailing site is a plateau-like hill at elevation of 60 m asl overlooking elementary and high schools built at the foot of the dumpsite that is surrounded by various ecosystems (e.g. mangrove, agricultural, river and marine) and communities that may pose risk to health and safety. The site is predominantly barren with patches of one to seven year old *Acacia auriculiformis*, *Saccharum spontaneum*, *Pityrogram macalomelanos*, *Muntingia calabura* and *Trema orientalis* and different ferns and very limited grass species.

The soil is acidic (pH 5.0, 1:1 soil: water) with low organic matter content (0.52%), available Bray P (92 ppm) and K (0.25 ppm). It contained Cu, Pb, Cd and Zn although only Cu exceeded beyond the maximum allowable limit of 36 mg/kg soil.

Collection of rhizosphere soil: Rhizosphere soil of about 20 plants including ferns and grasses growing in the 32ha abandoned mine tailing area after open pit mining for gold and copper were collected. The collected soil samples and the plants were brought to the laboratory for the isolation of mycorrhizal spores, quantify root colonization by mycorrhizal fungi and for isolation of NFB.

Isolation and mass production of mycorrhizal spores: Spores of AMF were isolated from 20 collected rhizosphere soil samples using the wet sieving technique (Brundrett et al. 1996). Nine out (AMF associated on five ferns and four grasses) of the 20 soil-plant samples based on prominent or prevalent spore types observed on each host plant, were selected and were mass produced from a single spore per container planted with bahia grass. Spores were harvested after four months, separated from the growing medium, counted and picked up individually under a stereomicroscope.

Assessment of root colonization by AMF: The root colonization of indigenous mine tailing mycorrhizal fungi were assessed from the ferns and grasses aside from spore count. In the mass production of single spore isolates, roots of bahia grass were also taken after four months growth in the greenhouse.

A 0.2 g plant⁻¹ fine root (< 0.2 mm diameter) was cleared with 10% KOH in water bath set at 90 °C for 30 minutes. It was cooled at room temperature and bleached overnight with 50% H₂O₂. H₂O₂ was replaced with 0.1 N HCl, rinsed with water and then stained with 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.

Commercial mycorrhizal inoculants: Mycorrhizal inoculants with trade name MYKORICH® and MYKOVAM® contain twelve species of AMF belonging to the genera *Glomus*, *Gigaspora*, *Acaulospora*, *Scutellospora* and *Entrophospora*. These were collected and isolated from stressed sites such as grasslands and abandoned mine tailing areas. MYKOVAM®, the first mycorrhizal inoculant developed at BIOTECH-UPLB in 1980, is effective in promoting growth and yield of a variety of crops including agricultural crops, fruit crops, ornamentals and forest species. Originally contains three AMF species: *Gigaspora margarita*, *Glomus etunicatum* and *Glomus macrocarpum*. In 2010, five more mycorrhizal species found effective in promoting growth of a variety of plant species were added into the MYKOVAM®. In this experiment, there are 12 species of AMF in the MYKORICH® and MYKOVAM® that comes in capsule or in powder form, respectively.

Preparation of narra seedlings: Pods of narra were collected from a genotypically superior plus tree growing at the UPLB campus. The seeds were manually extracted from the pod and then sown in trays filled with oven sterilized sand. Two weeks after germination, similar sized seedlings were planted into plastic cups (4 cm diameter at the rim x 2 cm diameter at the bottom) filled with oven sterilized soil and sand (1:1 v/v) mixture.

Inoculation of narra with AMF: Inoculation was done during planting in the plastic cups at the rate of 100 spores per seedlings per cup (4 cm diameter at the rim x 2 cm diameter at the bottom). The spore suspension was poured in a two inches deep hole at the center of the container where Narra seedling was inserted. After two months, seedlings were then transferred in polybags filled with two kg oven sterilized mine tailing soil (one seedling per polybag) obtained from Mogpog, Marinduque. During transfer, the soil sand ball of each seedling was left intact (not disturbed). Seven hundred fifty (0.75kg) g mine soil was placed at the bottom and 1.25 kg were added surrounding the narra seedling. The transferred seedlings were weighed before water was added to have a resulting weight of 2.75 kg. Growth was monitored for additional three months.

Experimentation: The screenhouse experiment was established following Randomized Complete Block Design (RCBD) with ten replicates. There were twelve treatments: AMF from five ferns and four grasses thriving in the mine tailing area. These were selected based on the number of spores produced in four months and with high percentage of root colonization on bahia grass. The growth promoting effect of the mine tailing indigenous mycorrhizal fungi were compared with MYKORICH® (coded as MRICH) and MYKOVAM® (coded as MVAM) -plus a control (no mycorrhiza).

Measured attributes

Seedling height and diameter. Shoot height and diameter of narra seedlings were measured once a month. Seedling height was measured one inch above the soil surface up to the tip of the main shoot using a ruler or a meter stick. Shoot diameter was measured one inch above the soil surface using a digital vernier caliper.

Seedling dry weight. Shoot and leaves were cut one inch above the soil surface, washed in running water to remove soil or dirt, air dried, wrapped separately in paper towels, placed inside brown paper bags and oven dried at 70°C for three days. Soil particles were removed from the roots by washing thru running water. Fine roots (≤ 0.2 mm diameter) were separated from the coarse roots by macerating the roots by hand and the detached fine roots were collected on a wire mesh screen (350 μm). Fine roots were blot dried to remove excess water, then 0.2 g samples were obtained for the assessment of mycorrhizal root colonization. Fine and coarse roots were separately wrapped with paper towels, placed in paper bags and oven dried similar to that of the shoot and leaves. Dry weights were measured using an analytical balance.

Spore count and mycorrhizal root colonization. Spore count and root colonization produced in the different mycorrhiza inoculation treatments were determined following the procedures stated above.

Copper concentration. Ground leaves, shoot and roots samples were analyzed by the College of Agriculture-Agricultural Systems Cluster Analytical Services Laboratory, UPLB, College, Laguna for Cu concentration. Cu concentration was analyzed in an atomic absorption spectrophotometer (AAS).

Statistical analysis

All data collected were analyzed using a one way analysis of variance (ANOVA) of RCBD. Treatment means were compared using Tukeys test at $p < 0.05$. Statistical analyses were done using MSTAT-C statistical computer program (Michigan State University 1996).

3 Results

Seedlings height and diameter

Mycorrhizal fungi associated with Fern2 consistently gave the tallest seedlings from 3 to 5 months after planting (data not shown), whereas control seedlings were generally the shortest throughout the 5-month observation period. Based on the height increase per month, the indigenous AMF from Ferns1, 2, and 5 had the

significant effect, which were comparable with the commercial MRICH and MVAM inoculants (Figure 1A).

AMF from Fern2 gave the largest seedling diameter from 2 to 4 months and was outgrown by MRICH-inoculated seedlings during the 5th month (data not shown). Indigenous AMF associated with Ferns1, 2 and 5 significantly higher shoot diameter increase per month of narra seedlings over the control, but had comparable with those observed in MRICH and MVAM inoculated seedlings (Figure 1B).

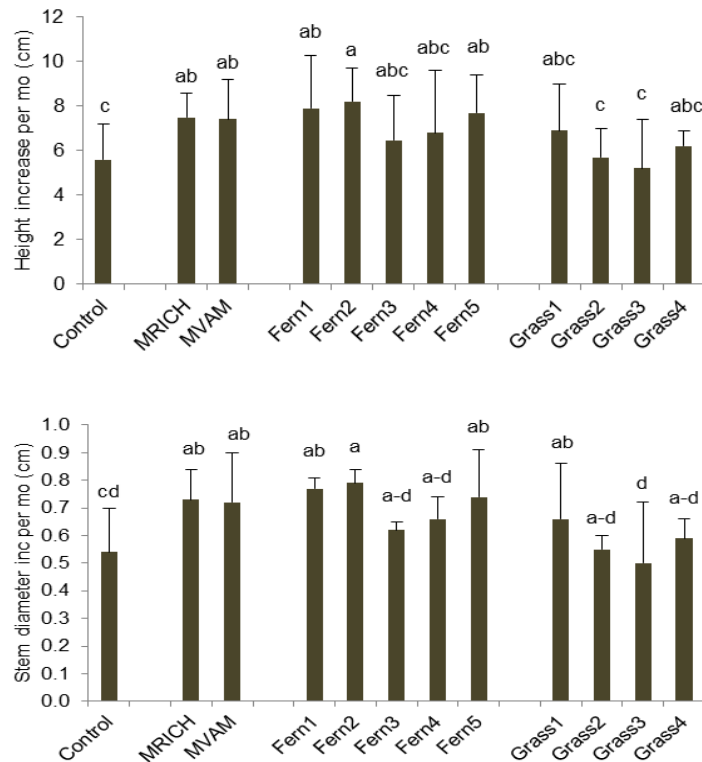


Figure 1. Monthly increase in height (A) and stem diameter (B) of five month old Narra seedlings grown in soil sand mixture (two mo) and then in mine tailing soil (3 mo) due to inoculation with mined-out indigenous mycorrhizal fungi and compared with a commercial mycorrhizal inoculant MYKOVAM®.

Seedling dry weight

All mycorrhiza inoculation treatments improved the dry weight of the roots over the control. Among the treatments, AMF from Grass1 provided the heaviest (3.89 g seedling⁻¹) roots, which increased by 209% over the control (1.26 g seedling⁻¹). Although Grass1 inoculated seedlings had heavier roots than the MRICH and MVAM inoculated ones, root dry weights were comparable from each other. Other four new mycorrhizal isolates (Fern2 and 3, Grass2 and 4) also had comparable root dry weight as compared with those inoculated with Grass1, MVAM and MRICH (Figure 2A). The uninoculated control seedlings had the lowest weights.

Nodule formation in the roots was also improved by the indigenous AMF inoculants based on the dry weight but some were comparable with the control despite the increase in weight. The root nodules were heaviest in seedlings inoculated

with MVAM followed by those inoculated with MRICH. Among the newly tested inoculants, those from Ferns2, 5, and Grass1 significantly improved root nodule dry weight of the narra relative to the control (Figure 2B).

The total seedling dry weight of the narra was all significantly increased by the mine tailings mycorrhizal isolates together with the commercially available AMF inoculants over the control. MVAM and Grass1 gave similar and the heaviest total seedling dry weight. The dry weight ranged from 3.5 to 15.2 g seedling⁻¹, whereas the lightest was observed in the control (Figure 2C).

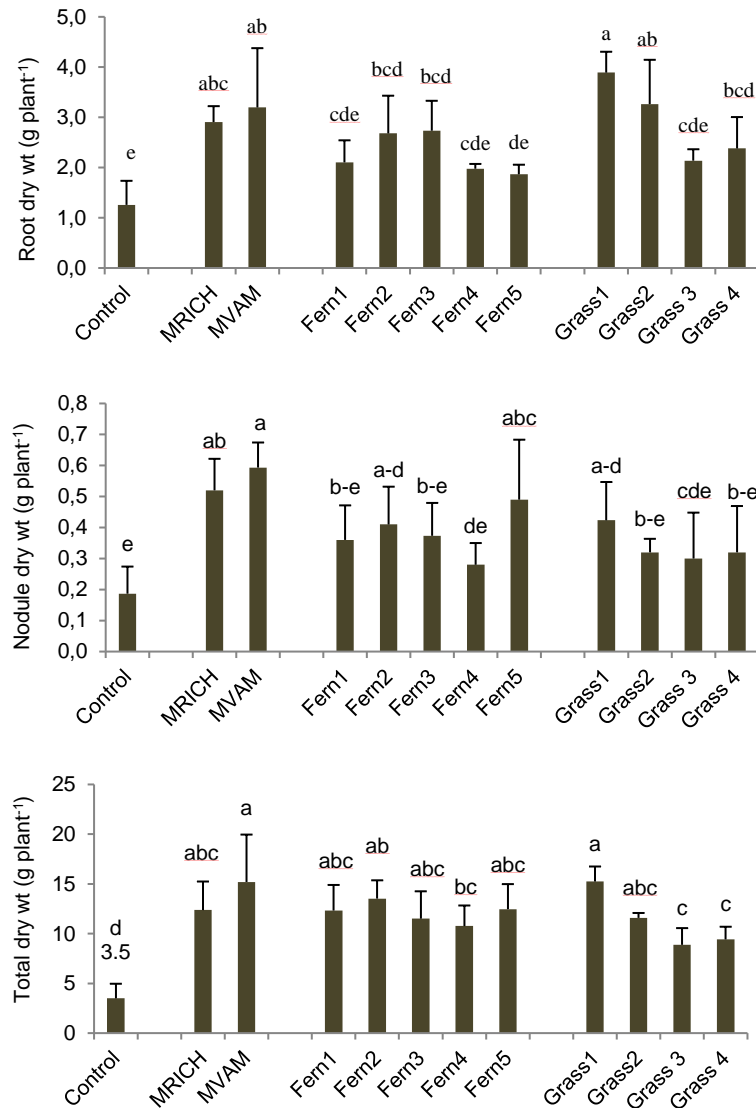


Figure 2. Partitioned root (A), nodule (B) and total dry weight of five month old narra seedlings grown in soil sand (2 mo) and then in mine tailing soil (3 mo) due to inoculation with mined-out indigenous mycorrhizal fungi and compared with a commercial mycorrhizal inoculant MYKOVAM®.

Spore count and mycorrhizal root

The number of spores in the rhizosphere and mycorrhizal root colonization of seedlings thriving in the mine tailing area in Barangay Capayang, Mogpog, Marinduque are shown in Figure 3. Two groups of seedlings were made, those seedlings with low spore count below 50 spores per 10 g sample (Figure 3A) and those with above 500 to 3000 spores per 10 g sample (Figure 3B). The latter are the ferns and likewise showed high percentage root colonization by mycorrhizal fungi (Figure 3C).

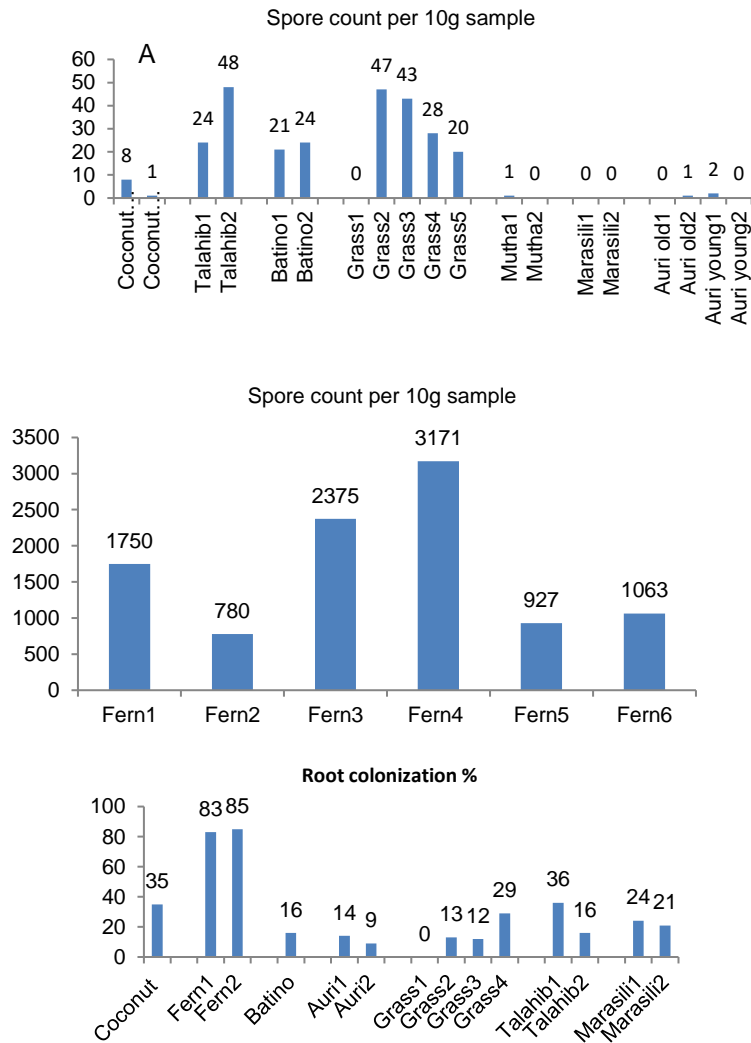


Figure 3. Comparative spore count and root colonization by mycorrhizal fungi on sampled plants thriving or planted in the mine tailing area in Barangay Capayang, Mogpog, Marinduque, Philippines.

On narra, all the AMF inoculation treatments gave significantly higher spore count (Figure 4A) and root colonization (Figure 4B). The control counterpart had the lowest number of spores $g\ soil^{-1}$ ranged from 2 to 20 spores $g\ soil^{-1}$, whereas the control had the lowest population counted (3 spores $g\ soil^{-1}$) (Figure 4A). Among the treatment, Fern2 provided the highest number of spores $g\ soil^{-1}$ but with inoculation

of AMF, introduced or native, significantly improved the number of spores in the contaminated soil compared with the control counterpart.

The percentage of mycorrhizal root infection level was doubled in mycorrhizal inoculated seedlings and significantly higher than the control. The infection rate of AMF ranged from 61-86%, whereas the uninoculated seedlings had the lowest percentage rate of 37%.

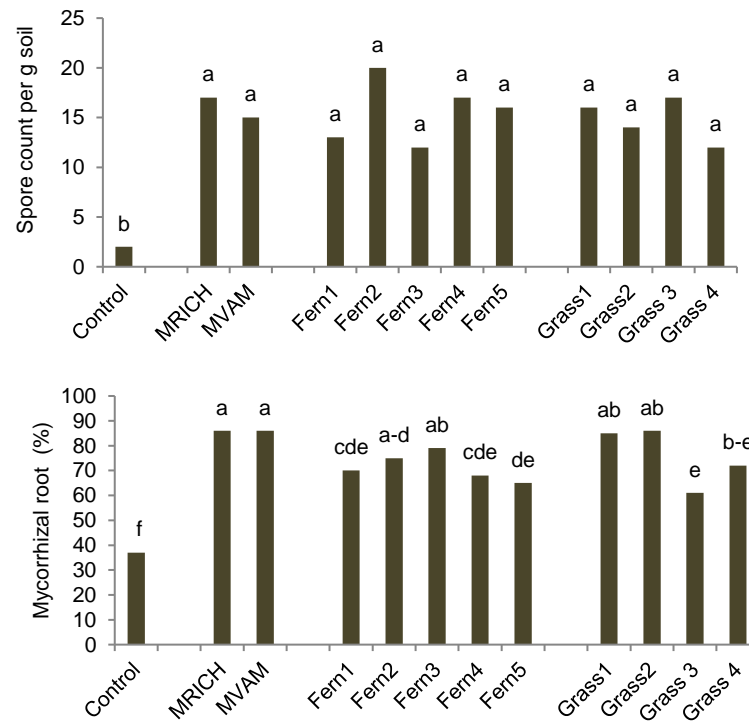


Figure 4. Mycorrhizal spore count (A) and root colonization (B) by mycorrhizal fungi on five month old Narra seedlings grown in mine tailing soil due to inoculation with mined-out indigenous mycorrhizal fungi and compared with a commercial mycorrhizal inoculant MYKOVAM®.

Cu accumulation in the roots, leaves and shoot

The Cu content in the roots, leaves and shoot of narra vary as they were grown in soil treated with different AMF inoculants. Cu accumulation was higher in roots compared to the shoot for all the treatments except for AMF from Grass4 (Figure 5A).

Among the inoculation treatments, Fern2 inoculated seedlings accumulated the highest Cu in the leaves (Figure 5B). Cu content in the leaves of Fern2 inoculated seedlings was also higher than those inoculated with MVAM and MRICH. However, Cu contents in the last two treated seedlings were comparable with those inoculated with Ferns4 and 5. Grass1 and Grass2 had the lowest Cu accumulation in the narra leaves (Figure 5B).

The Cu content in the shoot was comparable with each other in both seedlings inoculated with the indigenous AMF and the commercial AMF inoculants. Highest Cu accumulation in the shoot was found in Grass2 inoculated seedlings (Figure 5C) whereas those inoculated with AMF from Grass1 had the lowest (Figure 5C).

Finally, from the whole seedling, the roots had the highest amount of accumulated Cu, which reached up to >400 mg kg⁻¹ followed by Cu accumulated in the leaves and lowest in the shoot. Narra seedlings inoculated with mine tailing mycorrhizal isolates associated with Ferns2, 4, Grass1 and MYKOVAM[®] gave similar Cu concentration in the roots. Among the treatments, narra seedlings inoculated with AMF from Grass4 gave the lowest Cu accumulation in the roots (Figure 5C).

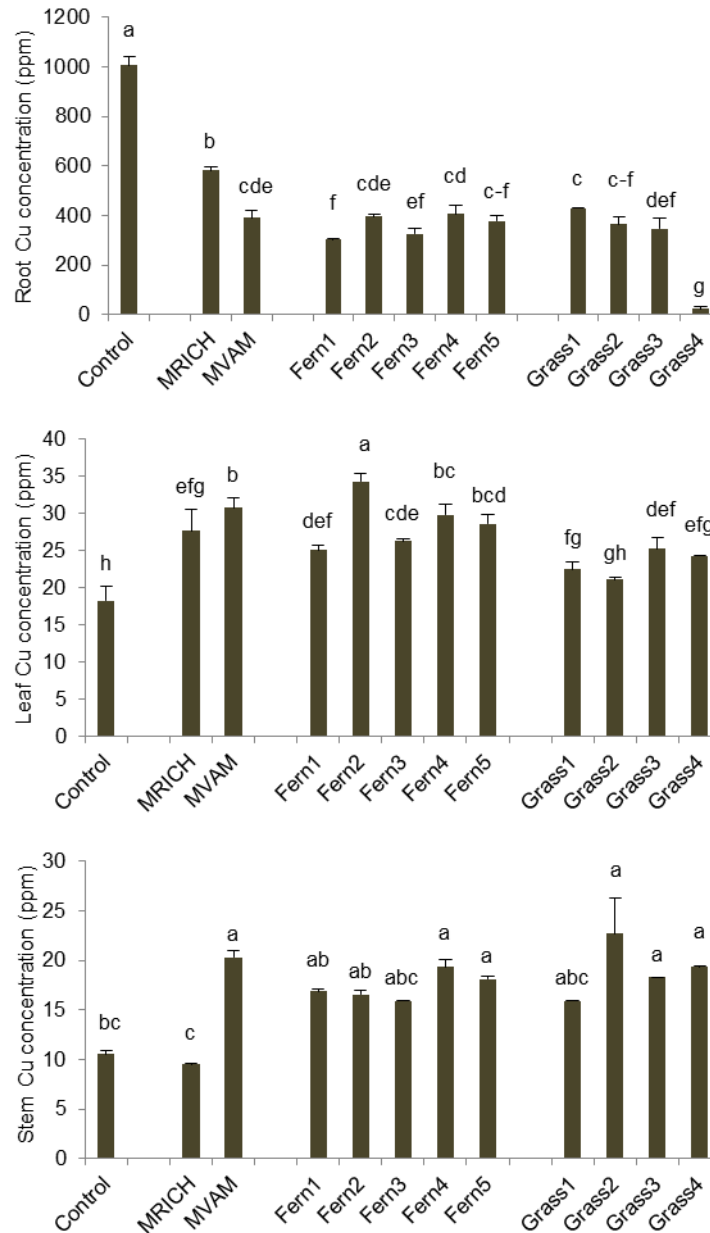


Figure 5. Partitioned Cu concentration in the root (A), leaves (B) and stem (C) of five month old narra seedlings in mine tailing soil due to inoculation with mined-out indigenous mycorrhizal fungi and compared with a commercial mycorrhizal inoculants MYKORICH[®] and MYKOVAM[®].

4 Discussion

Bioremediation strategies are significant in rehabilitating abandoned mining areas. This biotechnology application is widely adopted by using biological agents such as arbuscular mycorrhizal fungi (AMF) due to their sensitivity towards a range of soil pollutants (Leyval et al. 2002). AMF have repeatedly been demonstrated to alleviate heavy metal stress of plants (Hildebrandt et al. 2007) and develop physiological and morphological mechanisms to support plant growth response to various stresses such as salinity, drought, and heavy metals (Miransari 2017). This study used indigenous AMF, which were associated with indigenous ferns in the gullies and few grasses on the plateau of the mined-out areas. Several studies demonstrated the effectiveness of indigenous AMF in stimulating plant growth in heavy metal contaminated sites (Gildon and Tinker 1983; Diaz et al. 1996; Cabello 1999; Malekzadeh et al. 2011). Some studies had reported otherwise (Enktuya et al. 2000). In this study, majority of indigenous AMF isolates stimulated the height and shoot diameter of the narra seedlings when grown in contaminated soil. Based on the height and shoot diameter increase per month, indigenous AMF associated with Ferns 1, 2, and 5 had showed a best effect, which was comparable with the effect of the commercially available MYKOVAM® and MYKORICH®. Despite of the increase, not all indigenous AMF provided a significant effect on the height and shoot diameter suggesting that AMF-plant association may vary depending on the isolate and plant species used. Overall, the total seedling dry matter was stimulated by AMF, irrespective of the isolate origin. Our results are similar to Zhang et al. (2015) and Jiang et al. (2016) suggesting that AMF can improve the overall growth of the seedlings and enhanced the resistance to heavy metal-contaminated environment.

In addition, AMF also promoted heavier root nodules in the narra seedlings grown in heavy metal contaminated soil. Narra is considered as leguminous plant where associated rhizobia species induce nodule formation through root colonization (Wagner 2011). Nitrogen-fixation by these bacteria occurs in the root nodules (Van Kammen 1995). Moreover, NFB are considered as mycorrhizal helping bacteria, which enhance mycorrhizosphere in host plants through provision of reduced form of nitrogen (Azcón-Aguilar and Barea 2015). The result of this study may suggest a positive interaction between AMF and NFB by increasing the seedlings root nodule mass, which indicate to symbiotic improvement of the narra seedlings growth in heavy-metal contaminated soil. There are evidences that the interaction of AMF and plant-growth promoting bacteria (e.g. NFB) may lead to an integrated microbial process for the bioremediation of soil with heavy metals (White et al. 1998; Vivas et al. 2006; Gamalero et al. 2009; Mishra et al. 2015).

AMF communities vary between functional groups (legumes and nonlegumes), between plant species, and between parts of a root system (roots and root nodules) (Scheublin et al. 2004). Host plant species exerted a selective influence on AMF population size and diversity and the abundance of AMF are modified in metal-polluted soils (Del Val et al. 1999).

There are plant species that are not capable of establishing and growing in the mine tailings, regardless of microbial amendments, but studies showed that mycorrhizal plants had better growth in soil with or without contamination (Shetty et al. 1994). This study demonstrated that AMF alleviate heavy metal toxicity to the narra seedlings. The Cu concentration in the roots was about 10 times higher than the Cu

concentration in the shoot (except Grass4), suggesting that AMF inoculation induced Cu retention in the roots of the seedlings. These results corroborated with the earlier reports that AMF can alter the translocation pattern of heavy metals in the host plant (Shetty et al. 1994; Jiang et al. 2016). AMF adapt cellular mechanisms for their survival in Cu-contaminated environments, which include restriction of toxic trace levels into their cytoplasm, intracellular complexation of the metal in the cytosol and compartmentalization strategies (Ferrol et al. 2009). Specific metal transporters translocate Cu to the vacuoles causing a less damage of toxicity and at the same time Cu is accumulated into the specific fungal structures such as extraradical spores and intraradical vesicles (Ferrol et al. 2009). These responses are regulated by the heavy metal-dependent expression of different AMF genes in the intra- and extraradical mycelium (Hildebrandt et al. 2007).

On the other hand, the mycorrhizal population in the roots and rhizosphere of narra seedlings was improved upon application of AMF, regardless of the isolate origin. The increase of the percentage root infection by AMF increase with higher AMF soil spores density in the heavy metal-contaminated soil, which was similarly noted by Khan et al. 2014. In contrast, soil disturbances including increase heavy metal contamination often reduced the number of spores and root colonization by AMF (Waaland and Allen 1987), which can be attributed to the possible changes in the species diversity of mycorrhizal fungi when exposed to wide range of heavy metal contamination (Mozafar et al. 2002). However, the detection of AMF in uninoculated control, whether in the roots or soil, is due to the contamination from air and water introduced during the experiment.

5 Conclusion

Irrespective of isolate origin, AMF stimulated the total dry matter of the narra seedlings. The shoot and root growth were affected by the selected indigenous AMF isolates. Generally, AMF also prevented the translocation of Cu from root to shoot of the seedling. The mycorrhizal population of AMF also increased in the roots and rhizosphere upon treatment, regardless of AMF origin. The tested indigenous AMF generally improved the growth of narra seedlings grown in heavy metal contaminated soil and can be potentially developed to rehabilitate the abandoned mine tailings especially in Mogpog, Marinduque, Philippines. Indigenous isolates that had the best effect on the growth of narra seedlings in heavy-metal contaminated soil are subject for identification and can be used in bioremediating mined-out areas in the Philippines.

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