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Enhanced Rhizosphere Bacterial Population in an Abandoned Copper Mined-out Area Planted with *Jatropha* Interspersed with Selected Indigenous Tree Species

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ABSTRACT

Rehabilitation was conducted in an abandoned copper mined-out area in Mogpog, Marinduque by interplanting Jatropha curcas, a biodiesel source and potential phytoremediator, with different reforestation tree species, Pterocarpus indicus, Cassia spectabilis, Lagerstroemia speciosa and Bauhinia purpurea. The effect of the diversification treatment on the rhizosphere bacterial population, known to promote growth of host plant, was analyzed within a fifteen-month period (August 2007 to November 2008). The number of cultivable soil bacteria on-site prior to planting was very low (<0.01 to 2 x 10^3 CFU per g) owing to soil's acidity (pH 5) and poorly nourished condition. Higher bacterial population was observed from Jatropha rhizosphere than soil without vegetation. Bacterial population also varied with diversification treatments. Highest populations (7.3 x 10^4 to 1.3 x 10^5 CFU per g) of Cu-, Pb- and Zn-resistant bacteria were observed where in Jatropha was interplanted with Pterocarpus, Cassia, and Lagerstroemia. It appears that such treatment enhanced the population of heavy metal-resistant rhizosphere bacteria in Jatropha indicative of its potential in bioremediation. Randomly selected isolates were identified as Arthrobacter oxydans, Klebsiella variicola and Bacillus spp, which are all common soil bacteria. Rehabilitation of abandoned, mined-out areas can thus be naturally promoted by diversifying plants being introduced in such sites as this would enhance rhizosphere bacterial population.

Key words: *bioremediation, heavy metal-resistant bacteria, rhizosphere, Jatropha, interplanting*

INTRODUCTION

Mining remains to be a vital industry which can boost the economy of many countries. However, it is faced with environmental issues such as rehabilitation of abandoned sites for restoring biodiversity to improve ecosystem integrity (*Raymundo 2006*). Since mining activities damage land by removing the original soil, along with its organic matter and associated nutrients (*Wong 2003*), a post-mining rehabilitation program is essential.

A major problem in rehabilitation of degraded mining areas is the difficulty in growing back vegetation primarily due to extremely poor condition of the soil (*Wong 2003*). Numerous attempts to improve the capability of abandoned mined-out soils to support plant growth have been explored. These include augmentation of soil nutrients (*Walker 2008*) and enhancement of bacterial population of rhizosphere or the area surrounding the root system of the plant. These plant-associated microorganisms are known to promote the growth of host plants through increasing availability of essential nutrients like phosphate, nitrogen, manganese and iron, producing phytohormones, and providing protection against certain pathogens (*Osorio Vega 2007*).

Plants capable of growing in soils rich in heavy metals and metalloids (metalliferous) such as abandoned mined-out and mine tailings areas are likely candidates for phytoremediation, in which plants are used to bind and stabilize metals in its root system or even extract metal pollutants from soil (*Cadiz and Principe 2005*). Increasing evidences have shown that associated rhizosphere bacteria of phytoremediators play an important role in the accumulation and extraction of heavy metals from the environment by increasing bioavailability of metals in soil (*Li and Wong 2010*). Rhizosphere bacteria, particularly the heavy metal-resistant ones, also lessen the toxicity of heavy metal pollutants to plants by several mechanisms such as adsorption of metals on their cell walls (*Stout et al. 2010*), secretion of metal-chelating agents and transformation of toxic heavy metals to forms that are more readily taken up into roots (*Jing et al. 2008*). Moreover, rhizosphere bacteria can be good indicators of plant stability in stressed and polluted environments. A higher number and diversity can be associated to better plant growth (*Duineveld and Van Veen 1999*). Currently, the conventional inoculation technique used in bioremediating abandoned mines is both

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laborious and time-consuming. Thus, there is a need for a more natural and sustainable procedure for enhancing rhizosphere bacterial population for bioremediation.

Interplanting different species is already an accepted practice of improving plant performance, both in agriculture and reforestation activities (*Abouziena et al. 2010, Carpenter et al. 2004*). Its effect on the population of rhizosphere bacteria however, has not been adequately explored, especially in degraded, abandoned mined-out soils.

The present study was conducted in an abandoned copper-mined out area in Mogpog, Marinduque from August 2007 to November 2008. This area is considered as one of priority sites for rehabilitation by the Department of Environment and Natural Resources in order to stop or minimize further deterioration and dangerous effects of heavy metal contaminants to people living in nearby villages (*Goño 2007*). The study was aimed at evaluating the effect of planting *Jatropha curcas*, a possible source of biodiesel (*Arceo 2010, Kywe and Oo 2009, Lu et al. 2009*), in combination with several indigenous reforestation species on the microbial communities in the rhizosphere.

MATERIALS AND METHODS

Experimental Site

A two-hectare experimental site was identified by the Bioremediation Team of the National Academy of Science and Technology based on a field reconnaissance survey of the abandoned copper-mined out area in Barangay Capayang, Mogpog, Marinduque (*Raymundo et al. 2006*).

Experimental Design

The experiment was conducted following a Randomized Complete Block Design with four blocks, five treatments and ten replications. The five treatments were: Treatment 1 (no diversification), *Jatropha* seedlings alone; Treatment 2, *Jatropha* interspersed with *Pterocarpus indicus* (narra); Treatment 3, *Jatropha* interspersed with narra and *Cassia spectabilis* (anchoan dilau); Treatment 4, *Jatropha* interspersed with narra, anchoan dilau and *Lagerstroemia speciosa* (banaba); and, Treatment 5, *Jatropha* interspersed with narra, anchoan dilau, banaba and *Bauhinia purpurea* (alibangbang).

Soil Sampling and Chemical Analysis

Approximately 200 g of composite rhizosphere soil of *Jatropha* were collected from 10 to 20 cm deep around a ten cm radius using a trowel, three, six, twelve and fifteen months after planting on August 2007, hence covering both wet and dry seasons. The soil samples were kept at ambient room temperature during transport to the laboratory, after which these were either processed immediately or kept at 4 °C until testing.

Soil samples obtained from the site before transplanting were submitted for chemical analysis to the Central Analytical Services Laboratory of the National Institute of Molecular Biology and Biotechnology (BIOTECH, UP Los Baños).

Planting of *Jatropha* with Indigenous Reforestation Species

Seedlings of *Jatropha* and four other Philippine indigenous tree species (narra, anchoan dilau, banaba and alibangbang) that are fast growing and thus frequently utilized in local reforestation activities were planted in the experimental site with a block area of 50 m^2. Since *Jatropha* was the focus of a biofuel program of the Philippine government at the time of the study in addition to its medicinal value, it was planted under different diversification treatments as stated in the Experimental Design.

Jatropha and the reforestation species (about one-foot tall) were obtained from the nursery of the College of Forestry and Natural Resources at UP Los Baños and were brought to Mogpog, Marinduque. The seedlings were allowed to acclimatize to the prevailing environmental conditions for one month prior to planting in the field. All seedlings were treated with Mykovam™ (5 g per plant), lime (85 g per plant) and compost (100 g per plant) at the time of planting to promote the growth of the plants in the degraded soil on-site based on the study of *Aggangan and Aggangan* (*2012*). Lime and compost were mixed thoroughly with soil excavated from 100 cm^2 holes where seedlings were planted. Mykovam was placed directly beneath the roots. Seedlings were planted in a one-meter distance within a treatment, and two-meter distance between treatments. Re-application of 100 g compost was done to each seedling six months after transplanting to promote survival of the plants since chemical analysis of the soil from the study site revealed a very low amount of organic matter and other nutrients.

Determining the Population of Rhizosphere Bacteria

Ten g of soil sample were added to 90 ml of sterile phosphate buffered saline (0.85% saline and 0.1 M phosphate buffer). A serial ten-fold dilution was done and appropriate dilutions were inoculated onto Trypticase Soy Agar (*TSA, Zimbo et al. 2009*) supplemented with Nystatin (final concentration of 1,000 units per ml, Bristol-Myers Squibb Company) using the spot plating technique (*Herigstad et al. 2001, Miles and Misra 1938*) to analyze more samples while utilizing the least amount of materials. Heavy metal-resistant bacteria were selected by separately

adding nitrate or sulfate salts of Cd (final concentration of 1.6 mg L^{-1}), Cu (72.0 mg L^{-1}), Pb (170.0 mg L^{-1}) and Zn (280.0 mg L^{-1}) to the same medium, concentrations of which were twice the amount of acceptable metal contents of soil based on the Dutch standards (*Chen 2000*). Inoculated plates were incubated at ambient room temperature for two to three days or until colony growth was visible. All colonies were counted and colony-forming units (CFU) g^{-1} of soil (dry weight) were computed. Ten gram of each of the samples were oven-dried at 55°C for at least one week to determine the dry weight of soil.

Identification of Representative Bacterial Isolates

Well-isolated bacterial colonies were randomly selected, picked and purified by repeated streak-plating on TSA. After purification, cultural and morphological characterization was done (*Lattuada and McClain 1998*); (*Aygan and Arikan 2007*) together with production of toxin crystals and lecithinase activity by growing on Mannitol Yolk Polymyxin Agar (*Lattuada and McClain 1998*) to determine diagnostic features of bacterial isolates. All isolates were eventually identified by sequencing their amplified 16S rDNA (see below) and comparing the sequence with those in the Genbank databases (www.ncbi.nlm.nih.gov/BLAST/).

DNA Extraction, Amplification and Sequence Analysis

Each randomly picked bacterial isolate was grown in 25 ml Tryptocase Soy Broth (TSB) for 24 h at room ambient temperature from which genomic DNA was extracted using ZR Fungal/Bacterial DNA Kit™ (Zymo Research, California, USA) following the manufacturer's protocol. To amplify the 16S rRNA genes in the genomic DNA of each bacterial isolate, primers 341f (5'-GCCTACGGGAGGCAGCAG-3') and 926r (5'- CCGTCAATTCCTTTGAGTTT-3') (Muyzer, De Waal, and Uitierlinden 1993) and one µL of undiluted genomic DNA extract as template were utilized in a polymerase chain reaction (PCR). The PCR reagent mixture for 25 µl reaction that was utilized consisted of 1x PCR buffer, 0.8 to 1.5 mM $MgCl_2$, 0.5 µM mixed dNTPs, 0.1 µM of each primer and 0.5 U of Taq Polymerase (Invitrogen). The cycle conditions as suggested by *Ellis et al.* (*2003*) were tried and after several rounds of optimization, the final conditions were set as follows: initial denaturation and enzyme activation at 94°C for 5 min, 20 cycles of denaturation at 95°C for 1 min, annealing at 65°C for 1 min, decreasing the temperature by 0.5°C every cycle and extension at 72°C for 1 min, additional 10 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min; and, final extension at 72°C for 30 min. Purified PCR products were sent to Macrogen, Inc. (South Korea) for sequencing. The resulting 16S rDNA sequences were analyzed by alignment with the GenBank databases using the BLAST algorithm.

Statistical Analysis of Data

Data collected on the number of soil bacteria were analyzed by ANOVA of a Randomized Complete Block Design. Treatment means were compared using Duncan's Multiple Range Test at $P \leq 0.05$ if ANOVA showed significant effects (*Fry 1993*). Statistical analyses were done using SAS® software (version 9.1.3, SAS Institute, Inc., USA).

RESULTS AND DISCUSSION

Soil Chemical Analysis

The soil in general was acidic and marginal, as indicated by low organic matter and potassium contents (**Table 1**). These characteristics are typical of abandoned mined-out areas because of the topsoil that was removed during the process of opencast mining (*Ghose 2001*). Since the experimental site was intensively mined in the 60's to 70's for copper and gold (*Amadeo 2007*), it was stripped of its topsoil resulting to limited natural vegetation which consists mainly of patches of Japanese acacia (*Acacia auriculiformis*) and talahib (*Saccharum spontaneum*). Residual copper content was still high (**Table 1**), indicative of persistent contamination on-site. The amounts of other heavy metals were relatively low as compared to Dutch soil standards (*Chen 2000*), which could imply that Cd-, Pb- and Zn-contamination was not a particular current problem at the experimental site. Post-mining soil dumps could have varying chemical properties, but majority tend to lose their organic matter and essential elements like nitrogen, potassium and phosphorus through years of storage (*Ghose 2001*). The soil in the study site could have initially high amount of phosphorus since this element was not limiting.

Initial Soil Bacterial Population

The initial number of cultivable soil bacteria in the experimental site was barely over 2.0 x 10^3 CFU g^{-1} of soil (**Table 2**). Compared with common garden soil with total bacterial counts that could reach over 4 x 10^6 CFU g^{-1} (*Torsvik and Ovreas 2002*), the bacterial populations of the samples were on the whole very low. This could be attributed to the soil's low organic matter and potassium content, and relatively high concentration of Cu, on top of its low pH.

The presence of heavy metals in the culture medium allowed for selection of heavy metal-resistant bacterial species that initially thrive in the soil. The concentrations of heavy metals in the medium were higher than those found in the soil of the experimental site in order to select for resistant organisms that could be isolated and utilized for future bioremediation studies in areas where concentrations of different heavy metals are of higher levels.

Table 1. Chemical characteristics of soil from an abandoned mined-out site in Mogpog, Marinduque as compared to that of urban topsoil.

Parameter	Mean Value	Allowable Concentration
	Abandoned Mined-out Soil[a]	Urban Topsoil
pH	5.07±1.11	6.75[b]
Organic Matter, %	0.52±0.17	≥5[b]
P (Bray), ppm	103.12±74.27	>20 [b]
K, cmol·kg^{-1}	0.25±0.24	>0.375 [b]
Heavy Metal Content, mg·kg^{-1}		
Cu	70.97±14.30	36 [c]
Cd	0.03±0.01	0.8[c]
Pb	0.68±0.27	85 [c]
Zn	4.31±0.58	140 [c]

[a] Soil sample from this study analyzed by the Central Analytical Services Laboratory, BIOTECH
[b] Taken from *Koenig and Isaman* (*1997*) as cited by *Kruse* (*2007*)
[c] Taken from *Chen* (*2000*)

Table 2. Heterotrophic bacterial populations of soil from an abandoned mined-out site in Mogpog, Marinduque cultured under aerobic conditions as compared to that of garden soil.

Total Heterotrophic Bacterial Counts (x 10^3 CFU g^{-1}, dry weight)	
Abandoned Mined-out Soil[a]:	
Cd-resistant (1.6 mg L^{-1})	2.00
Cu-resistant (72.0 mg L^{-1})	<0.01
Pb-resistant (170.0 mg L^{-1})	0.50
Zn-resistant (280.0 mg L^{-1})	0.50
Total (no heavy metal supplement)	2.00
Garden soil, total[b]	4,000.00

[a] From this study
[b] Taken from *Torsvik and Ovreas* (*2002*)

There were very low populations of heavy metal-resistant species (**Table 2**), estimated at only about 5.0 x 10^2 CFU g^{-1} of soil for Pb- and Zn-resistant species while <10 CFU g^{-1} for Cu-resistant species. This low bacterial population could be due to the fact that not all microorganisms in the experimental soil could adapt to an environment with much higher metal concentrations that could interfere with their metabolism (*Bamborough and Cummings 2009, Crowley 2008*).

Cd-resistant bacteria, on the other hand, were relatively more abundant at 2.0 x 10^3 CFU per g soil, suggesting the possibility that the soil bacterial population is resistant to Cd. This apparent Cd-resistance of the cultivated bacterial population could be due to the concentration used in the medium which was still within levels tolerated by majority of known soil bacteria. *Ruizhang et al.* (*1990*) stated that the threshold tolerable concentration of Cd for many cultivated soil bacteria is around 5 mg L^{-1}. *Shentu et al.* (*2008*) reported that 0.5 to 1.0 mg kg^{-1} levels of Cd could even enhance the initial soil microbial population in environment particularly the gram-positive bacteria and fungi. *Stuczynsky et al.* (*2003*) also showed that soil biological activities, particularly dehydrogenases, can be stimulated by addition of 10 mg kg^{-1} Cd to soil. Many known soil microorganisms are inhibited only at a range of one to five mM concentrations (approximately 100 to 500 mg L^{-1}) (*Piotrowska-Sege et al. 2005*).

Soil is expected to contain a highly diverse community of microorganisms, the species composition of which varies with changes in soil biological properties, that in turn affect its long term chemical and physical properties and ability to support plant growth (*Crowley 2008*). It has been observed, however, that heavy metal contamination in soil negatively affects the microbial population, community structure, and microbial activities and processes (*Khan 2000*). At high concentrations, such as the case of Cu content of soil from the experimental site, toxic effects of metals result in reduced microbial population and could have altered rates of key biological processes that underlie ecosystem function. High amount of Cu, along with low pH of soil can also be considered as toxic to pioneer plants naturally growing on-site. Excess amounts of Cu are known to severely retard plant growth because of chlorosis, root-tip browning and oxidative stress that enhances formation of reactive oxygen species (*Hari Babu and Sudha 2011*). Limited vegetation would also result to limited amount of simple organic matter extruded to the soil, and this in turn could limit the number of surviving heterotrophic soil bacteria.

Rhizosphere Bacterial Population

The rhizosphere is well established to enhance the biomass productivity of microorganisms due to the presence of nutrients excreted from roots (*Wu et al. 2006; Watt et al. 2006*). In the present study, the number of soil bacteria was found to be enhanced in the rhizosphere of Jatropha. The rhizosphere bacterial populations were generally much higher than those obtained from the same

soil before transplanting (**Figure 1**). This is particularly true when the plants reached the age of at least 12 months. The estimated total bacterial population from rhizosphere soil of 15 month-old Jatropha from all treatments averaged to about 5.9 x 10^4 CFU g^{-1}. The heavy metal-resistant bacterial population showed the same trend, all illustrating remarkable increase in bacterial population when compared to that of soil without any vegetation.

Effect of Plant Diversification on Number of Heavy Metal-Resistant Rhizosphere Bacteria

Three months after planting, total heterotrophic bacterial populations from the rhizosphere of *Jatropha* varied with the diversification treatments. In the early stages of the experiment when the plants were only three months old (**Figure 1A**), the bacterial population was significantly highest ($P \leq 0.05$) with an estimated value of 3.2 x 10^4 CFU per g soil in the rhizosphere of *Jatropha* planted interspersed with three additional plant species, namely narra, anchoan dilau and banaba (Treatment 4). The rest of the treatments showed much lower bacterial populations. This trend suggested that rhizosphere bacterial population was highest where there was diversification. Interplanting, which is widely practiced in agriculture and forestry, has been shown to increase plant growth performance. By varying the type of plants in one particular area, soil enzyme and microbial compositions are also varied (*Li et al. 2007*). The leguminous nature of the plants interspersed with *Jatropha* is of particular importance since some of their associated rhizosphere bacteria are known to fix nitrogen, together with non-leguminous tree where free living nitrogen-fixing bacteria interact.

As with the total heterotrophic rhizosphere bacteria, the number of Cd-, Cu-, Pb- and Zn-resistant rhizosphere bacteria of three month-old *Jatropha* also showed an increasing trend with plant diversification up to a certain extent. The bacterial population was significantly highest ($P \leq 0.05$) from rhizosphere of plants in Treatment 4 with estimated values that range from 1.3 x 10^4 to 4.5 x 10^4 CFU per g soil, while those from other treatments with populations

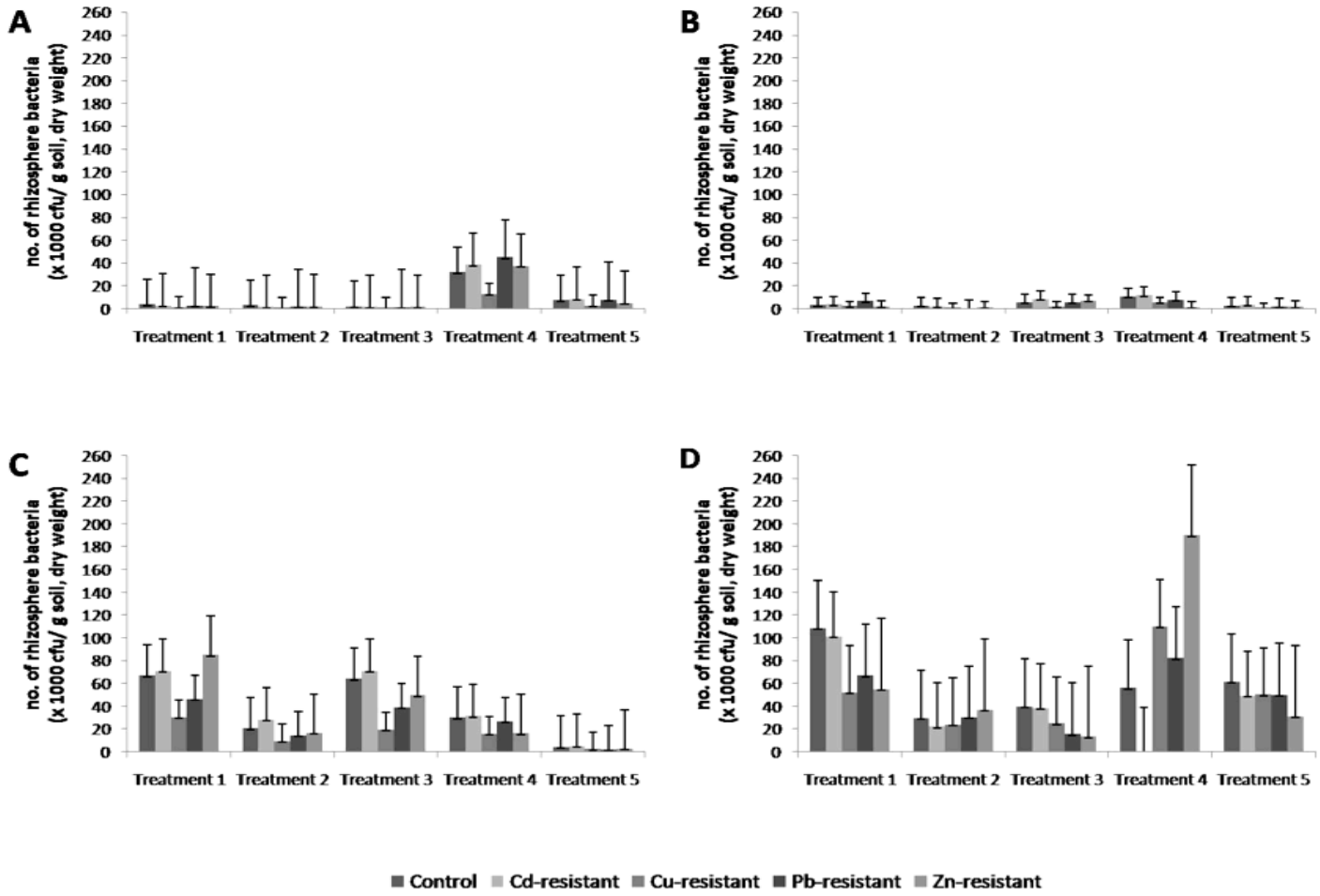

Figure 1. Estimated heterotrophic bacterial counts from rhizosphere of *Jatropha* planted in an abandoned mined-out site in Mogpog, Marinduque under different diversification treatments (Treatment 1 – all *Jatropha*, Treatment 2 – *Jatropha* interplanted with narra, Treatment 3 – *Jatropha* interplanted with narra and anchoan dilau, Treatment 4 – *Jatropha* interplanted with narra, anchoan dilau and banaba, Treatment 5 – *Jatropha* planted with narra, anchoan dilau, banaba and alibangbang) A. three months after planting; B. six months after planting; C twelve months after planting; D. fifteen months after planting.

as low as 6.0 x 10^2 CFU g^{-1} soil.

This trend of the increasing number of cultivable rhizosphere bacteria with plant diversity was not observed in Treatment 5, where in *Jatropha* was interplanted with four indigenous tree species (narra, anchoan dilau, banaba and alibangbang). This phenomenon could probably be due to increased interspecies root competition for nutrients and moisture (*Baumann et al. 2001*), or it could be due to a more complex interaction occurring among the plants and the soil microbial community. In a study by *Bartelt-Ryser et al.* (*2005*) on soil carry-over effects to plants and soil microbial diversity, the authors concluded that soils previously planted with legumes at high frequency have a more negative effect to growth of newly planted species than soils previously planted with legumes at low frequency. The authors partly explained this with the possibility of presence of species-specific soil-borne pathogens, such that these could have infected the new plants and negatively affected their growth. If this was the reason, the pathogens may have been carried in the roots of alibangbang; however, this was not observed in this study. The negative effect of legume frequency to microbial community structure, although detectable as well particularly with methods that detect microbial utilization of carbonic acids and alcohols, was considered only to be minor (*Bartelt-Ryser et al. 2005*).

Six months after planting. The number of rhizosphere bacterial population of *Jatropha* did not show significant differences among the various treatments six months after transplanting (**Figure 1B**). The previous trend of increasing number of cultivable rhizosphere bacteria of *Jatropha* with interplanting with leguminous and non-leguminous tree species was not observed. Populations of both total and heavy metal-resistant rhizosphere bacteria were low, ranging from 2.5 x 10^3 to 1.0 x 10^4 and 8.0 x 10^2 to 1.0 x 10^4 CFU g^{-1}, respectively. These observations of overall low rhizosphere bacterial population seem to suggest that *Jatropha* plants were still unable to duly colonize the Cu-contaminated soil on-site six months after transplanting. Plants that are well established in a particular soil normally contain higher microbial population in their rhizosphere compared to soil with no vegetation (*Watt et al. 2006*).

Twelve months after planting. A remarkable general increase in rhizosphere bacterial population was observed for *Jatropha* one year after transplanting (**Figure 1C**), which could imply that the plants increasingly became adapted and established on-site through time, especially since a full cycle of wet and dry seasons had passed. The total rhizosphere populations were significantly highest at an average value of 6.5 x 10^4 CFU g^{-1} from Treatments 1 and 3, where *Jatropha* was planted alone, and interplanted with narra and anchoan dilau, respectively. The heavy metal-resistant bacterial population shows the same trend, with average CFU g^{-1} of rhizosphere soil as follows: 7.1 x10^4 Cd-resistant members, 2.5 x 10^4 Cu-resistant members, 4.3 x10^4 Pb-resistant members and 6.2 x 10^4 Zn-resistant members. The positive effect of diversification treatments to rhizosphere bacterial population of *Jatropha* was not as much pronounced after 12 months since high populations were observed both for diversified and *Jatropha* alone treatments.

One noticeable general behavior of cultivable rhizosphere bacteria of *Jatropha* however, was the low populations observed for both total heterotrophic and heavy metal-resistant types in Treatment 5. Addition of alibangbang to the mix of indigenous tree species seemed to have elicited a negative effect to bacterial population in the rhizosphere of *Jatropha*. Although a definite conclusion regarding this observation cannot be made within the scope of this study, it is possible that indirect interactions did occur, such that alibangbang, a leguminous tree species reported to allocate more biomass to its roots (*Cai et al. 2007*), served as a better competitor in the poorly nourished, acidic and dry soil on-site and thus enhanced its own rhizosphere bacterial population, with a net negative effect to rhizosphere bacterial population of the surrounding vegetation including that of *Jatropha*. There is also the possibility that root exudates and foliage leachates of alibangbang could have negative effect to the rhizosphere bacteria of *Jatropha*. This allelopathic potential of alibangbang has been previously demonstrated (*Singh et al. 2009*) and was reported to be species-specific, such that inhibitory effect could only be observed in particular plant species that were interplanted with alibangbang.

Fifteen months after planting. Older *Jatropha* plants also exhibited varying rhizosphere bacterial population as affected by the plant diversification treatments. The total rhizosphere bacterial populations of 15-month old plants (**Figure 1D**) were significantly highest, with value of 1.1 x 10^5 CFU per g, in the control treatment where in no other plants were interspersed with *Jatropha*. Total heterotrophic bacterial populations from the other treatments were lower and of no significant differences at values ranging from 3.0 x 10^4 to 5.6 x 10^4 CFU g^{-1} of soil. Similar behavior and estimated counts were observed for Cd-resistant bacterial population. Interestingly, the population of rhizosphere bacteria that was resistant to at least 1.6 mg L^{-1} of Cd had apparent similar tendency with the total bacterial population as affected by the diversification treatments from all sampling time, again suggesting that all cultivable bacteria detected in the study were resistant to the concentration of Cd in the culture medium.

Cu-, Pb- and Zn-resistant rhizosphere bacteria from 15-month old Jatropha all showed comparable behavior as affected by the plant diversification treatments. Highest populations were observed from Treatment 4 with values ranging from 7.3 x 10^4 to 1.3 x 10^5 CFU g^{-1} of soil. Interplanting

Jatropha with narra, anchoan dilau and banaba apparently enhanced its Cu-, Pb- and Zn-resistant rhizosphere bacterial population, particularly during the initial plant colonization (after three months) and within 15 months after planting. Removal of banaba to the mix (Treatment 3) resulted to lower populations of 1.3 x 10^3 to 1.6 x 10^3 CFU g^{-1} for the said heavy metal-resistant bacterial group.

Overall, there was an observed positive effect of diversifying plant species to rhizosphere bacterial population of *Jatropha* in the abandoned mined-out soil of Mogpog. This general trend has been previously recognized in agriculture, where intercropping is claimed as one among the most significant techniques for a sustainable system (*Abouziena et al. 2010*). Its benefits include promoting land biodiversity, which encompasses improved soil enzyme activity and microbial population (*Chai et al. 2005*). *Abouziena et al.* (*2010*) explained that this dynamic increase of microorganisms in the rhizosphere of intercropped plants could be due to favorable quantitative and qualitative composition of organic compounds provided in the form of root exudates and crop residues or litter. Furthermore, this recognized nutrient facilitation among plant species as aided by rhizosphere microorganisms has been reported to increase in environments with higher levels of abiotic stresses (*Callaway and Walker 1997*). In extremely harsh conditions of mined-out soils that are usually very dry, acidic and contain very little nutrients, activity of soil microorganisms is of vital importance to plant productivity. Under such limiting conditions, up to 90 % of the P and N for plant growth might be provided by soil microbes (*Chen et al. 2008*).

The observed negative effect of adding alibangbang in the diversification treatment to the rhizosphere bacterial population of *Jatropha* in this study could be partly attributed to increased competition for nutrients and water in the marginal soils of the abandoned mined-out site with increased number of plant species, along with inherent competitive ability of each plant species used. An exceptionally good competitor would of course negatively affect growth and proliferation of poor competitors, which would also negatively affect the below ground community associated with those plants. One study of *Baumann et al.* (*2001*) showed a reduction in amount of yields and no increase even in biomass production of leek-celery intercrop system due to intra- and interspecific competition. The authors suggested maximizing the degree of complementarity between component crops and staggering relative planting time if needed.

In heavy metal-contaminated soils, the importance of enhanced population of heavy metal-resistant rhizosphere microorganisms is considered as one of the primary concerns in phytoremediation studies (*Jing et al. 2008*). This heavy metal-adapted group is in fact considered as a major contributory factor in phytosequestering and phytostabilizing metals in contaminated soil due to their ability to mobilize the contaminants making them available for uptake by plants (*Wu et al. 2007*), thereby increasing chances of phytoremediation success. Mobility of heavy metals can be improved by heavy metal-resistant rhizosphere bacteria through release of chelating agents, acidification, phosphate solubilization and redox changes (*Jing et al. 2008*). Thus, bacteria can augment the remediation capacity of plants, or at the very least, reduce the phytotoxicity of contaminated soils (*Stout et al. 2010*). Several studies in fact dealt with inoculating plants in heavy metal-contaminated soils with growth-promoting bacteria in order to increase their chances of survival and heavy metal uptake (*Kumari and Singh 2011, Darya et al. 2010, Ma et al. 2009, Sheng et al. 2008, Canbolat et al. 2006*). Moreover, soil remediation efforts not only aim to remove the metal contaminant by uptake but more importantly to restore the capacity of the soil to sustain vegetation in the long run and function according to its potential (*Chen et al. 2008*).

Identity of Representative Bacterial Isolates

Five representative bacterial isolates from the soil samples, randomly picked and purified, were successfully identified using combination of different approaches. Cultural growth of isolates 1 and 2 on TSA showed comparable light cream, mucoidal appearance with entire margin and convex elevation. Cellular morphology of these two isolates was very similar; both were rod-shaped and gram-negative. In contrast, isolates 3, 4 and 5 were all gram-positive, long rods. In addition, isolates 3 and 4 both were capable of producing endospores, while isolate 5 exhibited cellular pleiomorphism. These three gram-positive isolates showed varying culture appearance (**Table 3**).

Sequencing of 16S rDNA of two Zn-resistant isolates (isolates 1 and 2) showed 99 % similarity with that of *Klebsiella variicola* (**Table 4**), a relatively new Klebsiella species accepted in 2004 (*Brisse et al. 2006*) mostly associated with soil and plants, and has the ability to fix nitrogen. *Klebsiella* strains in general also produce capsule, mainly thick hydrophilic extracellular polysaccharides (*Brisse et al. 2006*) responsible for the glistening, mucoid aspect of colonies on agar plates (**Table 3**) and resistance of the organism to dry conditions in the environment, as exemplified by the abandoned mined-out soil in this study.

Spore-forming bacteria are likewise naturally present in soil of almost any type (*Madigan et al. 2012*) since endospores are quite resistant structures to majority of environmental stresses. Two species of *Bacillus* were identified from the abandoned mined-out soil of Mogpog, the Cu-resistant one (isolate 3) showing 100% 16S rDNA sequence similarity with *B. odysseyi* (**Table 4**). The Cd-resistant isolate (isolate 4), on the other hand, showed 100

Table 3. Morphological, cultural and physiological characteristics of selected metal-resistant bacterial cultures from soil of an abandoned mined-out site in Mogpog, Marinduque.

Isolate	Metal-Resistance	Cell Appearance	Gram Reaction	Colony Appearance	Lecithinase production
1	Zn	very short, straight rods	–	light cream, entire, convex, slightly sticky, mucoidal	nd
2	Zn	very short, straight rods	–	light cream, entire, convex, slightly sticky, mucoidal	nd
3	Cu	long rods, forms endospores	+	cream, entire, flat	nd
4	Cd	long rods, forms endospores, motile, no toxin crystals	+ +	white, opaque, large, dry, flat with irregular margin, no rhizoid	+
5	Cd	straight to curved rods		transluscent, glistening, entire, flat	nd

nd – not determined

Table 4. Identity of selected heavy metal-resistant bacterial isolates from an abandoned mined-out soil in Mogpog, Marinduque based on 16S rDNA sequence similarity in GenBank databases using BLAST algorithm.

Isolate	Organism With Sequences Of Significant Alignment	Genbank Accession	% Similarity*
1	*Klebsiella variicola* strain At-22	CP001891.1	99
2	*Klebsiella variicola* strain At-22	CP001891.1	99
3	*Bacillus odysseyi* strain 3P01SB	EU977788.1	100
4	*Bacillus cereus*		
	strain p2	HM004555.1	100
	LSBA1976	FJ908707.1	100
	5YW6	GU991861.1	100
	GXBC-1	GU982920.1	100
	LNTW72	GU969129.1	100
	NMSW50	GU969128.1	100
	B284	GU904693.1	100
	B275	GU904692.1	100
	JBS10	GU812900.1	100
	CPRI14	GU565569.1	100
	Q34	GU826154.1	100
	Bacillus mycoides strain SH2	HM037178.1	100
	Bacillus subtilis strain 0-2	FJ959367.1	100
	Bacillus thuringiensis		
	strain ucsc27	FN667913.1	100
	P22	GU826152.1	100
	JAM-GG01	AB553285.1	100
	Bacillus anthracis		
	strain CPRI13	GU565568.1	100
	H13	GU826150.1	100
	Q23	GU826153.1	100
	Bacillus pseudomycoides		
	strain J14	GU826151.1	100
5	*Arthrobacter oxydans* strain Mm2	GU391465.1	100

*E value is 0 for all presented alignments

% 16S rDNA sequence similarity with a group of *Bacillus* species known as the anthrax and pseudoanthrax bacilli with several members that are very closely related in terms of their 16S rDNA sequences (*Farrar and Reboli 2006*). The species members of this group though have varying phenotypic characteristics (*Farrar and Reboli 2006*) and therefore additional conventional characterizations could differentiate them. *Bacillus cereus* is motile with the ability to produce lecithinase. Unlike *B. thuringensis*, it cannot produce intracellular toxin crystals nor rhizoids in its colonies. Since isolate 4 has demonstrated the same diagnostic phenotypic attributes, it was therefore concluded that this particular isolate was *B. cereus*.

Another Cd-resistant isolate (isolate 5) from the abandoned mined-out soil sample was identified to be *Arthrobacter oxydans* based on 100 % similarity in their 16S rDNA sequences (**Table 4**). *Arthrobacter* belongs to the high Guanine-Cytosine actinomycete group of the gram-positive bacteria capable of fixing atmospheric nitrogen. *A.*

oxydans belongs to the globiformis group of arthrobacters, which exhibit typical pleiomorphism (**Table 3**). These organisms are usually considered to be the most numerically abundant cultivable, aerobic bacteria in the soil which could be due to their nutritional versatility and extreme resistance to drying and starvation (*Jones and Keddie 2006*), partially explaining their survival in the poorly nourished and dried condition of the abandoned Mogpog mined-out soil.

CONCLUSION AND RECOMMENDATIONS

The diversification of plants in heavy metal-contaminated soils leads to greater number of rhizosphere bacteria of *Jatropha* and may improve the growth and survival of this plant that has potentials for remediation. However, there seem to be a threshold as to the number and type of plant species that should be used, otherwise the population of rhizosphere bacteria tend to decrease. This could be related to increased competition for water and nutrients in marginal lands as the diversity of plant species, which have different competitive abilities, increases. There is also a possibility that alibangbang could negatively affect the number of rhizosphere bacteria of *Jatropha* because of inherent characteristics like higher root biomass that make them better competitor for soil nutrients and moisture. Likewise, alibangbang has been reported to have allelopathic potential, such that its foliage leachates and root exudates could have negative effect to the rhizosphere bacteria of *Jatropha*. In addition, the diversity of rhizosphere bacteria could also contribute to the differences in their abundance. Some species are naturally more tolerant to stresses and thus more persistent compared to other types. The possibility of augmenting certain rhizosphere bacterial population by inoculating growth-promoting cultures may also be explored. The identified heavy metal-resistant bacterial isolates from the abandoned Mogpog mined-out soil namely, *Klebsiella variicola*, *Bacillus cereus*, *B. odyssey* and A*rthrobacter oxydans*, could serve as a basis of what bacterial population in the soil could best be enhanced in order to increase the chances of plants' survival in metal-contaminated soils.

In conclusion, rehabilitation of abandoned, mined-out areas can be naturally promoted by diversifying plants being introduced in such sites as this would enhance rhizosphere bacterial population. Moreover, selection of reforestation species to be used in this diversification strategy must also consider competitive and allelopathic properties because these could disrupt phytoremediation process by negatively affecting growth of other plants. Maximizing the factors to enable self sustaining plant community establishment in these hostile soils are major concerns when success of phytoremediation researches is of prime intent.

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