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Taxonomic Microbiome Profiling and Abundance Patterns in the Cacao (*Theobroma cacao* L.) Rhizosphere Treated with Arbuscular Mycorrhizal Fungi and Bamboo Biochar

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Biochar and arbuscular mycorrhizal fungi (AMF) are agricultural interventions adopted by farmers to improve the growth of crops in nutrient-deficient acidic soil, which relatively influence the biological properties in the rhizosphere. This greenhouse study investigated the changes in prokaryotic diversity in the rhizosphere of cacao plants grown in acidic soil with AMF and bamboo biochar (BB) for 15 months under nursery conditions. Metagenomic analysis of the V3-V4 region of the 16S rRNA gene of the rhizosphere with AMF, 15% BB, and AMF + 15% BB revealed that the addition of AMF and BB reduced the sample's diversity, but the treatments increased the overall plant growth. Of all Operational Taxonomic Units (OTUs) recovered, the top three abundant phyla in the treated soils were: *Proteobacteria, Acidobacteria*, and *Actinobacteria*. The relative abundances of *Proteobacteria* and *Acidobacteria* increased with the treatments, whereas that of *Actinobacteria* was decreased. Biochar increased the unclassified genera under Order *Acidobacteriales* and *Chromatiales*. In contrast, AMF increased the abundance of an unclassified genus under Order *Xanthomonadales*. The genus *Rhodoplanes* was the most dominant in AMF + 15% BB-treated soil with a relative abundance of 9.4%. The four samples shared only 4.9% of the total OTUs; apparently, the microbial communities across samples were separated and distinct. Most of the increased genera have been reported to play essential roles in nutrient cycling, which may contribute to cacao growth promotion.

Key Words: arbuscular mycorrhizal fungi, bamboo biochar, cacao rhizosphere, prokaryotic community structure, 16S Metagenomics

Abbreviations: AMF – Arbuscular Mycorrhizal Fungi, BB – bamboo biochar, OTUs – Operational Taxonomic Units, PGPR – Plant Growth Promoting Rhizobacteria

INTRODUCTION

Cacao (*Theobroma cacao* L.) is an economically important crop for chocolate and cocoa production (Argout et al. 2011). Nowadays, the increasing global demand for chocolate and cocoa products triggers farmers to apply soil amendments and interventions to improve crop production. These interventions can improve plant growth and increase yield, especially in nutrient-deficient acidic soil, and act as an alternative to chemical fertilizers and other inorganic farming inputs. Biochar amendment (Baronti et al. 2010; Biederman and Harpole 2013) and arbuscular mycorrhizal fungi (AMF) inoculation (Smith et al. 2011; Roger et al. 2013) are examples of these interventions, which have been observed to enhance agricultural production and nutrient cycling, affecting the physical, chemical, and biological properties of the plant rhizosphere.

Biochar is a pyrolyzed carbon-rich solid product derived from certain biomass (Elad et al. 2011; Woolf et al. 2010; Lehmann and Joseph 2015). Biochar enhances the

soil's water-holding capacity and fertility, improves crop productivity, and is reported as a cost-effective means of carbon sequestration (Lehmann et al. 2011). The provision of nutrients for the growth of this crop is commonly driven by important soil microorganisms, which are primarily involved in nutrient cycling (Chen et al. 2013; Lu et al. 2016). The microbial community in the soil is commonly altered by biochar resulting in the increase and decrease of their relative abundances (Ducey et al. 2015; Khodadad et al. 2011; Kolton et al. 2011), depending on the soil type and condition (Aislabie and Deslippe 2013; Schreiter et al. 2014). Biochar-amended soil also promotes plants' systemic resistance to various foliar pathogens (Elad et al. 2011). On the other hand, AMF are obligate biotrophs that infect the plant's root system, essentially forming a symbiotic relationship and facilitating the uptake of essential nutrients such as phosphorus and nitrogen (Smith et al. 2011). This group of fungi has also been reported to shift the microbial communities and composition in the soil (Solís-Domínguez et al. 2011; Akyol et al. 2019). AMF act as a biological control by preventing the infection of disease-causing root pathogens and can increase plant tolerance to drought, salt stress, herbivores, and heavy metals (Sullia 1991; Smith and Read 2010; Yang et al. 2014). The promising effect of these agricultural options may be improved through their interaction. Biochar may serve as a refuge for various colonizing microorganisms, including AMF protecting them from soil predators (Warnock et al. 2007).

The bacterial community in the rhizosphere plays vital roles in facilitating the growth of various plants by exerting plant growth biocontrol effects (Elad et al. 2011). These rhizosphere-inhabiting microorganisms compete for water and essential nutrients and become competitive to establish a symbiotic relationship with the host plant (Hartmann et al. 2009). Plant-microbe interactions induce the rhizosphere effect due to the release of organic materials from the plant that provides a driving force in developing active microbial populations in the rhizosphere (Morgan and Whipps 2001). Many rhizosphere microorganisms regulate plant growth promotion and disease suppression (Meena et al. 2017) through nitrogen fixation (Igiehon and Babalola 2018), phosphate solubilization (Mehta et al. 2015), siderophore production (Sayyed et al. 2013), phytohormone production (Maheshwari et al. 2015), and secretion of secondary metabolites acting as biocontrol agents (Compant et al. 2005). The selective effect of the roots towards the selection of microbes also extends towards the root-associated and symbiotic fungi, such as mycorrhizal fungi (Hartmann et al. 2009). Root exudation, water and nutrient uptake by roots, decay, respiration, and physicochemical changes in soil influence the microbial composition and function in the rhizosphere (Lladó et al. 2017).

As part of the nutrient cycling, it is important to know the group of rhizospheric microorganisms that play vital roles in soil processes and the growth of the cacao plant. To the best of our knowledge, no information has shown the effects of biochar and AMF on the prokaryotic community structure in the cacao rhizosphere, specifically in an acidic soil environment. Thus, this study assessed the effects of bamboo biochar and AMF on the prokaryotic diversity and its composition in the cacao rhizosphere through high throughput next-generation sequencing.

MATERIALS AND METHODS

Greenhouse Experiment Preparation and Planting

The soil used in this study was collected in the marginal area of Cavite-Lumot-Cavinti Watershed (Cavinti, Laguna, Philippines) and used as the substrate for the cacao experiment. The soil was brought to the laboratory, air-dried, passed in a 2-mm pore opening wire mesh, and then stored in clean sacks for further analyses. Soil samples for physicochemical analyses were submitted to the Central Analytical Service Laboratory (CASL) of the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB). The biological analyses were done at BIOTECH and the Institute of Biological Sciences, UPLB. The results of the initial physicochemical analyses have been reported in the study of Aggangan et al. (2019a).

A nursery trial was performed in a screenhouse of the Mykovam Laboratory of BIOTECH, UPLB. The screenhouse was enclosed with a concrete structure at the foot of welded wires lined with fine nets to prevent the entry of insects and covered with plastic roofing to prevent cross-contamination due to splashing of rain droplets during the rainy season. The average temperature in the screenhouse was about 27.5°C. Experimental seedlings were placed on a steel bench with 1 m width, 5 m length, and elevated half a meter above the ground.

The biochar derived from bamboo trimmings and AMF inoculants were prepared as described by Aggangan et al. (2019b). Briefly, a total of 300 g of powdered biochar was mixed with 1700 g of acidic soil to achieve the 15% concentration of soil: biochar substrate. The substrate mixture was placed in black polyethylene

bags (4" x 10" x 2") (in triplicates), watered to field capacity (30 % moisture content), and incubated for two weeks prior to planting. For AMF inoculation, a soil-based powder AMF inoculant containing a mixture of 12 species belonging to the genera *Glomus, Gigaspora, Entrophosphora,* and *Acaulospora*, was used. The inoculant was developed and commercially produced at BIOTECH, UPLB, which contains 100-150 spores per gram soil, chopped mycorrhiza infected roots of bahia grass (85-100% colonization), and other infective propagules.

Cacao (UF18) seeds were obtained from cacao growers in Davao, Philippines. Seeds (with radicle) were sown immediately in oven-sterilized (100°C for 72 h) garden soil-sand (1:1) mixture and incubated for two weeks under nursery conditions. Healthy cacao seedlings were selected and transferred into the poly bags filled with (1700 g soil sand + 300 g biochar) substrate mixture previously cured for two weeks. Prior to transferring the seedlings into the pots, AMF inoculant was placed in a 2-3 inches deep hole at the center of the substrate at 5 g per seedling. Cacao seedlings were then transferred into the substrate in contact with the inoculated AMF. Control seedlings were also prepared but no AMF.

Experimental design and treatments

The experiment was laid out in two factors in RCBD with three replicates (one pot is considered as one replicate). The treatments were the following: untreated acidic soil (A1); AMF-inoculated soil (A2); 15% biochar-amended soil (A3); and combined AMF-inoculated and 15% biochar -amended soil (A4). The experiment was observed for 15 months under nursery conditions.

Collection of samples

After 15 months, rhizosphere samples were collected aseptically by cutting the pot in half, exposing the roots and rhizosphere of cacao plants. Four subsamples (5 g each) of the rhizosphere in each pot were randomly collected using a sterile spatula and clean gloves, mixed with the subsamples from the replicated pots to achieve composite samples for each treatment, placed in a sterile plastic bag, and immediately processed for community DNA extraction.

After harvest, other parameters were recorded and analyzed: the growth parameters of the harvested cacao plants, the percentage of AMF infection rate in the fine roots, and the physicochemical properties of the soil.

Cacao Plant Growth, AMF Root Infection, and Physicochemical Analyses

During harvest, three plants per treatment were collected and the whole plant parts of each plant were cut, separating the leaves, stems, and roots. Individual plant parts were washed, blot-dried, covered with tissue paper, placed in a brown paper bag, and oven-dried at 60°C for three days. The dry weight of the cut parts was measured using an electronic weighing scale (Mettler Toledo GmbH, Switzerland). Height and stem diameter increments were calculated by subtracting the final height from the initial height of the seedlings, which was measured immediately after transplanting in the substrate. Nutrient uptake of the cacao plant was calculated by multiplying plant nutrient concentration (%) of the young fully expanded leaf to the total wt. of dry matter (g).

For AMF infection rate, a total of 1 g of fresh fine roots were randomly collected from each plant as a replicate, cleaned in running water, and placed in the test tube with 50% ethanol. Roots were cleared and stained with trypan blue, placed on the petri dish with grid lines, and observed using a stereomicroscope (Olympus, Japan). AMF propagules in infected roots were counted in each grid, and the percentage of AMF colonization in ten grids was calculated based on the formula used by Ishii and Kadoya (1994).

During harvest, a total of 500 g of soil were collected and sent to the CASL, BIOTECH, UPLB for the physicochemical analyses following the protocols described by Aggangan et al. (2019b).

Statistical Analyses

The data on the plant growth, AMF root infection rate, and soil physicochemical properties were analyzed using two-factor ANOVA in RCBD with three replicates. Treatment means were compared using LSD if ANOVA showed significance at $P \leq 0.05$. Statistical analyses were performed using MSTATC program version 2.1.

Community DNA Extraction

A total of 460 mg of composite soil from each sample was used for DNA extraction using FastDNA® SPIN Kit for Soil (MP Biomedicals, France) with minor modifications as follows: (1) addition of 40-mg skim milk (Takada-Hoshino and Matsumoto 2004) and 460-mg soil sub-sample into a Lysing Matrix E tube before the addition of lysing buffers; (2) addition of three washes as follows: first wash used equal volume of binding matrix and 100%

ethanol into the supernatant that was then mixed by inversion for 12 min using a Mini LabRoller™ Rotator (Labnet International, USA) before transferring to the SPIN™ filter, second wash used 300 µl of binding matrix + 300 µl of 100% ethanol placed into the SPIN™ filter, and third wash used 500 µl of 100 % ethanol before the step of adding SEWS-M for final washing; and (3) DNA sample was eluted using 60 µl pre-warmed (55°C) DES (DNase/ Pyrogen-Free Water).

Extracted DNA was run on 1% TAE agarose gel at 100 V for 60 min. The purity and concentration of DNA were determined using nanodrop and fluorometric method (Qubit 2.0).

PCR Amplification of V3 – V4 Region of 16S rRNA Gene

Polymerase chain reaction (PCR) analysis was performed by Apical Scientific Sdn Bhd (Selangor, Malaysia) using 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') (Yu et al. 2005) primer set that targeted the V3 - V4 region of the 16S rRNA gene. A 45-µl reaction was carried out using Phusion® High-Fidelity PCR Master Mix (New England Biolabs, USA) under the following conditions: initial denaturation at 94°C for 2 min, followed by 20 cycles of 98°C for 10 sec, annealing at 51°C for 30 seconds, and 68°C for 1 min. The quality and quantity of PCR products were measured using TapeStation 4200, Picogreen, and Nanodrop. The integrity of DNA was verified using gel electrophoresis by running 1 µl of PCR product on 1.7% TAE agarose gel at 100 V for 65 min.

Library Preparation and Sequencing

Sequencing libraries were generated by Apical Scientific Sdn Bhd (Selangor, Malaysia) using TruSeq® DNA PCR-Free 16S metagenomics library preparation kit (Illumina, USA). The resulting libraries were validated qualitatively using Agilent TapeStation 4200, followed by an assessment of library concentration (ng μl^{-1}) using a Qubit@ 2.0 Fluorometer (Biotek FLX800). The resulting NGS libraries were validated quantitatively with real-time PCR (qPCR) and pooled for demultiplexing and sequencing quality control. Purified libraries were sequenced using the Illumina MiSeq platform at 2x301PE format (Illumina San Diego, CA, USA).

Bioinformatics Analyses

The data preprocessing and taxonomic classification of the sequence reads from the four samples were carried out in a QIIME pipeline (Quast et al. 2012). Paired-end reads in FastQ format were first removed of index and adaptor sequences using BBDuk of the BBTools packages (https://sourceforge.net/projects/bbmap). Briefly, the primer and barcodes were removed, and the forward and reverse sequence paired reads were merged to form one continuous DNA sequence using FLASH v2 and quality screened for sequence length and nucleotide ambiguity. Ambiguous sequence tags with bases less than 150 bp in length or with homopolymers of greater than 600 bp (sequenced on the MiSeq platform) were removed from downstream processing. Sequence reads were then aligned with the 16S rRNA database and inspected for chimeric (unnatural) errors, which were subsequently screened using USEARCH against the RDP_GOLD v9 database and removed from downstream processing. After these quality assessment steps, reads were clustered at 97% similarity into Operational Taxonomic Units (OTUs) by default; rare OTUs with only 1 (singleton) or 2 reads (doubleton), which are often spurious, were deleted from downstream processing. The remaining sequences were aligned and classified using the SILVA rRNA reference database, and microbial diversity was analyzed using QIIME software (Quast et al. 2012).

Alpha diversity indices were measured using Chao1, Shannon, Simpson, and the total number of observed species (Shannon 1948; Simpson 1949). These indices were calculated based on 97% identity during sequence-clustering by default with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Species richness based on OTUs were compared through rarefaction curves (Gotelli and Colwell 2011). Beta diversity based on weighted UniFrac was calculated by QIIME software (Version 1.7.0). Two-dimensional Principal Coordinate Analysis (PCoA) was displayed by WGCNA package, using the stat and ggplot2 packages in the R software (Version 2.15.3). Moreover, the OTU abundance was normalized by converting it into a ratio (specific OTU count over the total OTU counts in a sample) to perform Correspondence Analysis (CA) using R packages (Le et al. 2008; Wickham 2016; R Core Team 2018; Kassambara and Mundt 2019).

RESULTS

Plant Growth, AMF Root Infection, and Soil Physicochemical Properties

The height and stem diameter of the cacao plants were consistently improved by biochar amendment regardless of AMF inoculation. The total plant dry wt. of the treated cacao plants is heavier than the control, but the mycorrhizal (+AMF) cacao plants grown with bamboo biochar demonstrated the heaviest total dry matter. The

dry wt. of the fine roots increased with AMF alone over the other treatments, including the control. The treatments generally improved the nutrient uptake of cacao plants and specifically the mycorrhizal cacao plants grown in acidic soil with biochar showed the highest N uptake. In addition, the infection rate of AMF was prominent in mycorrhizal plants and was further improved when biochar was combined. Moreover, the availability of essential minerals in the soil was generally improved by biochar amendment, whereas the combination of AMF and biochar demonstrated the highest available P and K in the soil (Table 1).

Richness and Diversity Analysis of Operational Taxonomic Units (OTUs)

A total of 4096, 3549, 2941, and 3531 OTUs were recovered in untreated soil (A1), AMF-inoculated soil (A2), 15% BB-amended soil (A3), and AMF + 15% BB-amended soil (A4), respectively. Alpha diversity showed that in terms of species richness (Chao1 and number of observed species), the untreated control (A1) showed the highest value, followed by treatments with AMF and BB (A4), AMF treatment alone (A2), and lastly by the biochar -amended soil (A3) (Table 2), suggesting that the treatments reduced the species richness of acidic cacao rhizosphere. Similarly, Shannon's and Simpson's indices revealed that untreated soil was more diverse than any of the treated soil samples. Chao1 values were higher than in the observed number of species, indicating that the values in the samples were underestimated. Also, the rarefaction curves on species diversity showed that the number of OTUs still increased with the number of sequences, without reaching a plateau.

Microbial Community Structure of Cacao Rhizosphere

Of all OTUs recovered, the top 3 abundant phyla in the untreated acidic soil were *Proteobacteria*, *Actinobacteria*, and *Firmicutes*, but members of the phylum *Firmicutes* were reduced in treated soils and dominated by the phylum *Acidobacteria* together with *Proteobacteria* and *Actinobacteria* (Fig. 1).

In all samples, the dominant phylum was *Proteobacteria*, but this phylum relatively increased in abundance in soils treated with the AMF with or without BB. Four main classes were observed within this phylum, including *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria*. The largest bacterial group *Alphaproteobacteria* increased in soil with AMF + 15% BB (21.6%), 15% BB (18.0%), and AMF (17.6%) over the untreated control (17.4%) (Fig. 2). Under *Alphaproteobacteria*, the most abundant genera (with > 1% relative abundance) observed across treated samples were *Rhodoplanes* (Order *Rhizobiales*), *Kaistobacter* (Order *Sphingomonadales*), and unclassified genus under Order *Rhodospirillales*. Genus *Rhodoplanes* was the most abundant in the acidic soil treated with AMF + 15% BB (Table 3). Meanwhile, AMF and bamboo biochar increased the relative abundance of *Gammaproteobacteria* (Fig. 2). At the order level, members of this group were abundant in cacao rhizosphere, including *Xanthomonadales* and *Chromatiales*, which were increased individually by AMF and

Table 1. Cacao growth traits, AMF root infection, and soil physicochemical properties as affected by arbuscular mycorrhizal fungi (AMF) and bamboo biochar (BB) after 15 days under nursery conditions.

	Plant Growth Traits							Soil Physicochemical Properties				
Treatment	Height (cm)	Stem Diameter (cm)	Fine Roots Dry wt. (g plant^{-1})	Total Plant Dry wt. (g plant^{-1})	N Uptake (g plant^{-1})	P Uptake (g plant^{-1})	AMF Root Infec-tion (%)	pH	N (g kg^{-1})	P (mg kg^{-1})	K (me 100 g soil^{-1})	OM (g C kg^{-1})
Control	89.55 c	1.13 b	3.54 b	61.31 c	1.25 c	0.08 b	18.67 c	4.78 a	0.60 c	3.90 c	0.22 c	12.8bb
AMF	76.85 d	1.26 b	9.95 a	100.63 b	2.05 ab	0.15 a	55.67 ab	4.70 a	0.50 c	3.65 c	0.25 c	9.50 b
15% BB	99.25 b	1.77 a	3.12 b	104.35 b	1.70 b	0.19 a	42.67 bc	4.90 a	2.20 a	16.75 b	1.34 b	78.70 a
AMF+15% BB	104.47 a	1.71 a	4.22 b	134.13 a	2.45 a	0.19 a	79.00 a	4.80 a	1.80 b	26.85 a	2.85 a	20.30 b

AMF – Arbuscular mycorrhizal fungi; BB – bamboo biochar; N – Nitrogen; P – Phosphorus; K – Potassium; OM– Organic matter.

Table 2. Alpha diversity analyses of the prokaryotic community structure in the cacao rhizosphere treated with arbuscular mycorrhizal fungi (AMF) and/or bamboo biochar (BB) after 15 days under nursery conditions.

Sample	Sample Name	No. of OTUs	Observed Number of Species	Chao 1	Shannon	Simpson
A1	No treatment	4096	3764	3981.258	6.708202	0.996041
A2	AMF	3549	3139	3403.183	5.944007	0.981468
A3	15% BB	2941	2649	2868.756	5.355482	0.978122
A4	AMF + 15% BB	3531	3148	3438.153	6.074773	0.992946

AMF – Arbuscular mycorrhizal fungi; BB – bamboo biochar; OTUs – operational taxonomic units.

biochar, respectively. Moreover, *Betaproteobacteria* was more abundant in untreated soil (4.1%) than the treated soils (0.7-3.1%), whereas the relative abundance of *Deltaproteobacteria* slightly varied in all samples.

Similarly, phylum *Acidobacteria* increased in abundance in all treated soils (11-15.1%) compared to the untreated counterpart (8.1%) (Fig. 1). Of all the classes of this phylum, *Acidobacteriia, Acidobacteria-6, Chloracidobacteria,* and *Solibacteres* were abundant with > 1% relative abundance (Fig. 2). This study observed that the bacterial Order *Acidobacteriales* (Class *Acidobacteriia*) dominated the cacao rhizosphere that was treated with 15% BB, whereas orders *Solibacterales* (Class *Solibacteres*) and RB41 (Class *Chloracidobacteria*) were generally increased by AMF regardless of biochar application.

In contrast with phyla *Proteobacteria* and *Acidobacteria*, the phylum *Actinobacteria* appeared to have decreased in abundance in all treated soils (Fig. 1). Three classes of Phylum *Actinobacteria*, including *Thermoleophilia, Actinobacteria, Acidimicrobiia*, were abundant in the cacao rhizosphere, but the treatments reduced their relative abundances. Despite the overall decrease in class *Thermoleophilia*, the relative abundance of Order *Solirubrobacterales* was increased by biochar regardless of AMF inoculation.

Moreover, the relative abundance of the phylum *Chloroflexi* was higher in untreated (9.6%) than in the treated samples (4.8-6.5%). Likewise, phyla *Firmicutes, Cyanobacteria,* and *Gemmatimonadetes,* which commonly include phototrophic bacterial species, decreased in the treated acidic soils over the untreated control (Fig. 1). Particularly, biochar amendment reduced the abundances of both phyla *Firmicutes* and *Gemmatimonadetes*, whereas AMF reduced the abundance of phylum *Cyanobacteria*. The most abundant genus in the cacao rhizosphere was *Bacillus* (Phylum *Firmicutes*), but their abundance decreased due to the amendment of biochar and inoculation of AMF (Table 3). Similarly, phyla *Verrucomicrobia* and *Bacteroidetes* also decreased their abundance with biochar application, but their relative abundances increased with inoculation (Fig. 1). In addition, phylum *Planctomycetes* considerably decreased with biochar amendment. Lastly, candidate phyla *TM7* and *AD3* increased in relative abundance in treated soil over the control (Fig. 1).

The archaeal phylum *Crenarchaeota* was detected in the cacao rhizosphere and represented by members of the class *Thaumarchaeota*. The relative abundance of this class increased in biochar-amended soil but was reduced in AMF-inoculated soil over the control (Fig. 2),

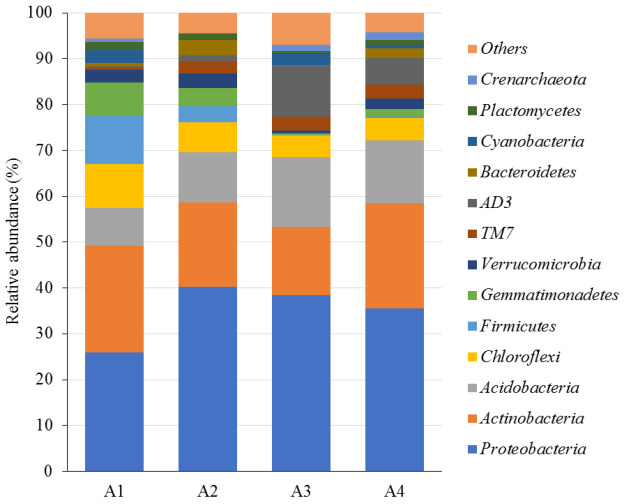

Fig. 1. Microbial community compositions at phylum level. The relative abundance (>1%) of each phylum in the total taxon tags in both bacteria and archaea are represented in each color. Samples include A1 (control); A2 (AMF alone); A3 (15% BB alone); and A4 (AMF+15% BB).

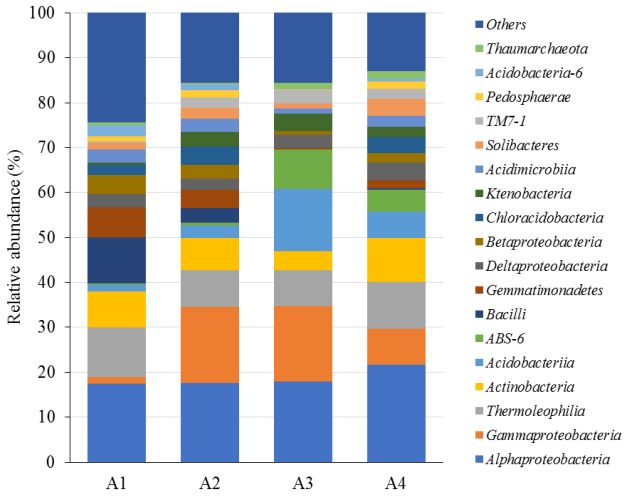

Fig. 2. Microbial community compositions at class level. The relative abundance (>1%) of each class in the total taxon tags in both bacteria and archaea are represented in each color. Samples include A1 (control); A2 (AMF alone); A3 (15% BB alone); and A4 (AMF+15% BB).

suggesting a vital role of biochar in improving archaeal diversity regardless of the AMF activity. Meanwhile, the unassigned group (*Others*) was also observed in all samples, indicating the presence of novel species of bacteria and archaea in the cacao acidic rhizosphere (Fig. 1).

The heatmap showed that the overall relative abundances of different phyla were altered by the treatments as indicated by color intensities and depicted by vertical and horizontal clustering.

Table 3. Dominant bacterial genera identified using high throughput 16S rRNA gene sequencing with ≥1% relative abundance.

Bacterial Genera	% Relative Abundance			
	Control (A1)	AMF (A2)	15% BB (A3)	AMF + 15% BB (A4)
Order Rhizobiales *Rhodoplanes*	5.7	3.8	5.8	9.4
Order Bacillales *Bacillus*	7.7	2.6	0.1	0.1
Order Sphingomonadales *Kaistobacter*	1.1	2.5	1.2	1.7
Order Nitrososphaerales *Candidatus Nitrososphaera*	0.7	0.3	1.3	1.7
Class ABS-6 Unclassified Genus	0.3	0.7	8.7	4.9
Order Acidobacteriales Unclassified Genus	0.1	1.1	0.4	3.1
Unclassified Genus	1.2	1.4	13.3	2.6
Order Solibateres Unclassified Genus	0.7	0.7	0.6	2.6
Order RB41 Unclassified Genus	1.4	2.0	0.008	1.8
Order Acidomicrobiales Unclassified Genus	1.8	2.9	1.1	2.3
Order Actinomycetales Unclassified Genus	0.3	2.4	2.2	2.7
Order Gaiellales Unclassified Genus	7.3	5.6	3.3	6.3
Order Solirubrobacterales Unclassified Genus	2.4	0.7	0.3	0.5
Unclassified Genus	0.3	0.8	3.1	2.9
Order Thermogemmatisporales Unclassified Genus	0.2	2.6	2.1	1.7
Order Ellin5290 Unclassified Genus	1.7	2.6	0.1	1.0
Order Rhizobiales Unclassified Genus	0.9	1.8	2.2	1.0
Order Rhodospirillales Unclassified Genus	3.9	4.1	8.3	5.9
Order Myxococcales Unclassified Genus	0.9	1.8	1.9	2.3
Order Chromatiales Unclassified Genus	0.001	0.0	11.5	1.5
Order Xanthomonadales Unclassified Genus	0.8	1.7	3.7	2.9
Unclassified Genus	0.3	14.4	0.1	1.3
Others (<1% contribution) *	60.3	43.5	28.7	39.8

AMF—Arbuscular mycorrhizal fungi; BB—Bamboo Biochar
*Includes all identified genera with <1% relative abundance.

Relationship Between Microbial Communities

Based on weighted UniFrac PCoA, the microbial communities in A1, A2, A3, and A4 were distinct and separated from each other according to PC1 and PC2 (51.22% and 32.02% variation explained, respectively) (Fig. 3). Along PC1, A3 and A4 were grouped to the right of the graph, whereas A1 and A2 grouped to the left of the graph. In addition, along PC2, A2 and A4 grouped to the top, whereas A1 and A3 at the bottom of the graph. Overall, the UniFrac PCoA results demonstrated distinct prokaryotic communities of cacao rhizosphere across samples as they are distributed in separate quadrants (Fig. 3). This study further showed a clear shift in the rhizosphere microbial community structure of cacao seedlings, especially when grown in soil with biochar alone because the microbial community, based on the relative abundances, clustered away from the other treatments, including the control (Fig. 4).

The soil microbiome across the four samples exhibited some unique (separate section) and shared (overlap section) microbiome profiles shown in a Venn diagram (Fig. 5). The microbiome of cacao rhizospheric soil (A1), AMF-inoculated soil (A2), biochar-amended soil (A3), and combined AMF-inoculated and biochar-amended soil (A4) showed 685 commonly shared OTUs,

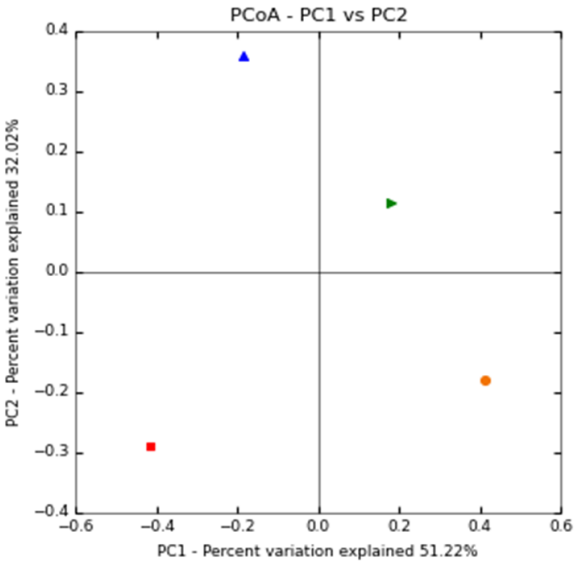

Fig. 3. Principal Coordinate Analysis (PCoA) based on weighted UniFrac metrics for A1 (untreated control soil, red square), A2 (AMF-inoculated soil, blue triangle), A3 (biochar-amended soil, orange circle) and A4 (AMF-inoculated and biochar-amended soil, green triangle) was indicated. Samples were collected after 15 months from planting.

which was about 4.9% of the total OTUs. Samples A1, A2, A3, and A4, have 1475, 610, 593, and 439 unique OTUs, respectively (Fig. 5), suggesting the distinct microbial community structure and composition across samples as affected by biochar and mycorrhizal fungi.

Besides, several unique OTUs were observed in the untreated control (A1), which include the genera *Lysinibacillus, Rummeliibacillus, Sporomusa, Balneimonas, Acidovarex, Chitinomonas, Anaeromyxobacter, Myxococcus, Hydrocarboniphaga, Prosthecobacter,* and others. In the treated samples, genera *Coprobacillus, Sphingobium,* and *Methylibium* were some of those unique OTUs in the rhizosphere with AMF alone (A2), whereas genera *WCHB1-84* (family *Peptococcaceae*), *YNPFFP6* (family *Sulfobacillaceae*), and unclassified genus (order *Chromatiales*) were unique in the biochar-amended soil (A3). Lastly, unique OTUs in the cacao rhizosphere treated with AMF and biochar (A4) included the genus *Rhodanobacter* and unclassified genera under the family *Aerococcaceae* and *Phyllobacteriaceae* (data not shown).

DISCUSSION

Cacao (*Theobroma cacao* L.) plant is an important crop to produce chocolate and other cocoa products. However, due to its poor agronomic performance and disease susceptibility, agricultural options were adopted by farmers, such as the introduction of hybrid genotypes of cocoa varieties to produce fine flavor chocolate (Argout et al. 2011) and application of soil conditioners and biofertilizers to improve *T. cacao* growth (Aggangan et al. 2019b).

The Philippines is seen to have a competitive advantage in *T. cacao* production in Asia, but its overall production shared less than 0.01% in the global market

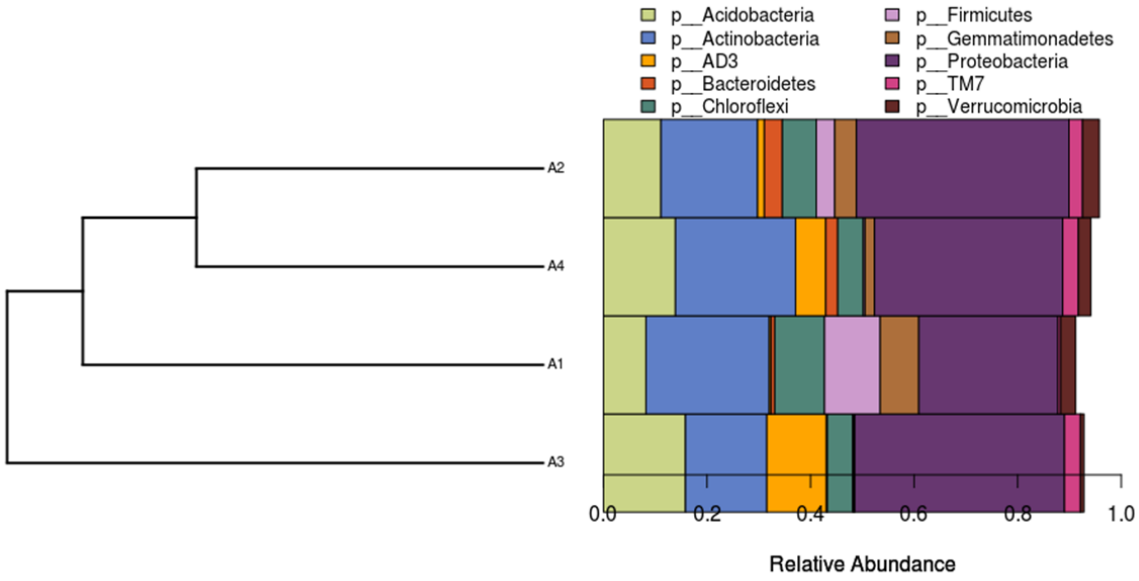

Fig. 4. Weighted UniFrac UPGMA cluster of microbial communities associated with different cacao rhizosphere samples. The relative abundance indicates the percentage of the specific phylum in the total taxon tags of each sample. Samples include A1 (control); A2 (AMF alone); A3 (15% BB alone); and A4 (AMF+15% BB). The relative abundances of the 10 most dominant phyla in all samples are indicated, and the rests are indicated as 'Others'.

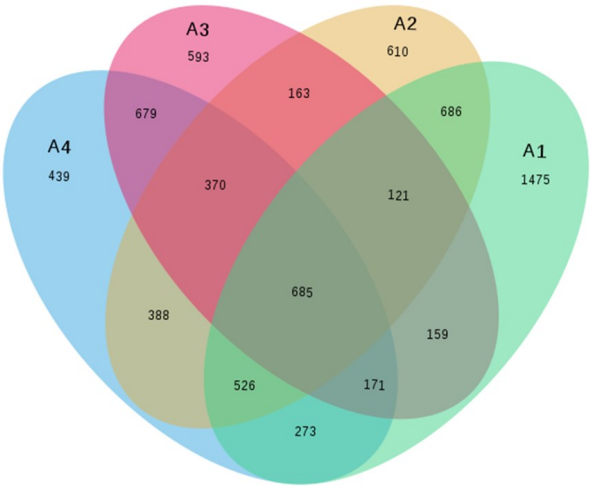

Fig. 5. Venn diagram of the OTUs in four cacao rhizosphere samples showing unique and shared OTUs based on 97% similarity. The number inside the diagram indicate the numbers of OTUs. Samples include A1 (control); A2 (AMF alone); A3 (15% BB alone); and A4 (AMF+15% BB).

(Department of Trade and Industry 2017). This limitation can be attributed to certain factors, such as inadequate access to advanced agricultural practices and farming techniques, production and postharvest facilities, and a viable and sustainable market for the product in the Philippines (Quilloy 2015). This study could help fill the gaps on the weakness of cacao production in the country because the results demonstrated the beneficial effects of bamboo biochar amendment and AMF inoculation on the overall growth of *T. cacao*. Biochar provides nutrients and improves nutrient solubilization and uptake by plants, thus enhancing plant growth and resistance against pathogenic microorganisms (Graber and Elad 2013). On the other hand, AMF, which is associated with about 80% of terrestrial plants, exhibits a positive relationship with plants by enhancing nutrient uptake and growth, increasing plant survival, ensuring plant re-establishment, and improving soil structure (Wang 2017). This study observed the synergy between bamboo biochar and AMF because their combination produced the highest values on the height, overall dry matter, N and P uptake, AMF root infection, and available P and K in the soil. Warnock et al. (2007) summarized the mechanisms on how biochar augments the symbiotic relationship between the AMF and plants: (i) biochar changes soil nutrient availability that affects AMF activity; (ii) biochar alters the activity of other microorganisms that have effects on mycorrhizae; (iii) biochar alters the signaling dynamics between plants and mycorrhizal fungi or detoxifies allelochemicals; and (iv) biochar serves as a refuge for colonizing fungi and bacteria. This study corroborated the first mechanism by which the addition of biochar increased the nutrient availability in the nutrient-poor soil, particularly the N, P, K, and OM, resulting in the increased root colonization by mycorrhizal fungi. Observations made by Ishii and Kadoya (1994) also support this result. The rest of the mechanisms suggested by Warnock et al. (2007) have something to do with the effects of biochar and AMF on the soil microbiota, which is the primary goal of this study.

Based on the alpha diversity, the species richness in the treated samples was lower than the control, and AMF and bamboo biochar reduced the diversity indices. This result contradicted the results in the biochar-amended soil of Kolton et al. (2017) and AMF-inoculated soil of Gui et al. (2017). The addition of biochar to soils results in alterations with beneficial or detrimental effects to other soil microorganisms (Warnock et al. 2007). Also, the relative effects of the treatments on the species diversity may depend on the nature of the soil and plants used. Besides, despite the decrease in species richness and diversity, this study observed an increase in the overall plant growth. This growth-promoting effect could be attributed to the possible enrichment of beneficial microorganisms, such as those mycorrhizal helping bacteria, which operate nutrient cycling in the rhizosphere (Warnock et al. 2007; Azcón-Aguilar and Barea 2015).

The bacterial species belonging to the dominant phyla *Proteobacteria, Acidobacteria,* and *Actinobacteria* in treated soils could play vital roles in the cacao rhizosphere ecology, as these phyla were already found to be dominant in other acidic soil environments (Rousk et al. 2010). Of all the classes of phylum *Proteobacteria*, the class *Alphaproteobacteria*, which includes plant growth-promoting rhizobacteria (PGPR) (Fernández-Gómez et al. 2019), was the most abundant in the treated samples. This observation corroborates with the results of Akyol et al. (2019) and Anderson et al. (2011), suggesting that amendment of AMF and biochar increase the abundance of these common symbionts (*Alphaproteobacteria*) of plants. This group can become established in either symbiotic or nonsymbiotic plant-association (Pini et al. 2011) by processes that involve nitrogen-fixation, which converts atmospheric nitrogen (N_2) into ammonia (Lindström et al. 2015), and processes related to P turnover (Lladó et al. 2017). At the genus level, Gkarmiri et al. (2017) also demonstrated the dominance of genera *Rhodoplanes* and *Kaistobacter* in the plant rhizosphere, which play important roles in plants such as nitrogen-fixation and degradation of aromatic compounds, respectively.

Similarly, Fernández-Gómez et al. (2019) observed that the alphaproteobacterial genus *Kaistobacter* was abundant and enriched in the rhizosphere of native plants, which was identified as a plant-disease suppressor genus (Liu et al. 2016). Also, the species under class *Gammaproteobacteria* were suggested as potential health indicators of healthy banana plants on Fusarium wilt-infested fields (Köberl et al. 2017), including the species under orders *Xanthomonadales* and *Chromatiales*. Besides, Maltz et al. (2019) demonstrated that *Xanthomonadales* is the most abundant bacterial Order in soil that received acidic treatments and based on our results, it is the most abundant in acidic soil inoculated with AMF. This group possibly established a positive association with mycorrhizal fungi because of the mycorrhizosphere as also suggested by Lladó et al. (2017). The family of *Chromatiales* is commonly photosynthetic and primarily involved in sulfur cycling (Imhoff 2014). Moreover, all known ammonia-oxidizing bacteria belong to *Gammaproteobacteria* and *Betaproteobacteria*, which responded positively with acidity, pH, and ammonia availability of the soil (Lladó et al. 2017). Overall, the growth improvement of cacao plants as subjected to AMF inoculation and biochar amendment could be partially attributed to the increase in abundance of certain PGPR.

Similar to the Phylum *Proteobacteria*, the Phylum *Acidobacteria* also increased in abundance due to the treatments. Members of Order *Acidobacteriales* (Class *Acidobacteriia*) dominated the biochar-amended cacao rhizosphere, which are suggested to have essential roles in biogeochemical processes with their extensive metabolic versatility and adaptation to low pH and varying substrate availabilities (Naether et al. 2012). An increase in the relative abundance of phylum *Acidobacteria* upon biochar amendment corroborates with the study of Gao et al. (2017). Also, Rousk et al. (2010) reported that, in arable soil, there are subgroups of *Acidobacteria* that were relatively increased and decreased with increasing soil *pH* environments, which may suggest that the alkaline nature of biochar could elevate the abundance of certain acidophilic microorganisms and their abundance help the cacao plants cope in the acidic nature of the soil.

The phylum *Actinobacteria* exhibits an enormous diversity in morphology, physiology, and metabolic capabilities (Barka et al. 2016). Despite the decrease, the members of this group have been reported to secrete a wide range of antimicrobial compounds (Bulgarelli et al. 2013) and degrade/decompose all sorts of organic substances (Anandan et al. 2016). Meanwhile, Santana et al. (2016) demonstrated that the members of bacterial Order *Solirubrobacterales* are categorized as endophytic microorganisms, which established an intimate relationship with the root tips and leaves of *Baccharis dracunculifolia*. One novel endophytic species, *Solirubrobacter phytolaccae*, was isolated from the root system of the *Phytolacca acinosa* Roxb (Wei et al. 2014). This study demonstrated the positive effect of bamboo biochar in increasing the population of endophytic *Solirubrobacter* species, which could enhance the growth of cacao plants.

The pattern of increase of both phyla *Proteobacteria* and *Acidobacteria* and decrease of phylum *Actinobacteria* in biochar-amended soil were similarly noted by Gao et al. (2017), suggesting that biochar can alter the community structure of common symbionts in the plant's rhizosphere. In contrast, Gui et al. (2017) demonstrated a different trend where AMF decreased the relative abundance of *Acidobacteria* and increased *Actinobacteria*.

The presence of the organohalide-respiring bacteria (Phylum *Chloroflexi*) in the cacao rhizosphere may play an important role in detoxifying the anthropogenic groundwater contaminations in the soil (Hug et al. 2013). On the other hand, *Bacillus* (Phylum *Firmicutes*) secrete several metabolites that trigger plant growth and prevent pathogen infection as well as improve plant productivity under unfavorable climatic conditions (Radhakrishnan et al. 2017).

A decrease in the relative abundance of *Gemmatimonadetes* in biochar-treated soil was similarly noted by Xu et al. (2016). However, some studies contrastingly reported that biochar induced the relative abundance of bacteria within the phylum *Gemmatimonadetes* (Khodadad et al. 2011). Members of the phylum *Verrucomicrobia* have been reported to oxidize methane. Some are better adapted to an extremely acidic environment (Dunfield et al. 2007); thus, the decrease in their relative abundance might be attributed to the alkaline nature of bamboo biochar. The candidate division *TM7* is ubiquitous and an uncultured phylum of bacteria associated with acidic soil environment (Winsley et al. 2014).

These overall shifts of the prokaryotic community structure observed in this study corroborated with the previous studies, suggesting that the microbial diversity and composition were altered following the application of biochar (Khodadad et al. 2011; Kolton et al. 2011; Jenkins et al. 2017) and arbuscular mycorrhizal fungi (Solís-Domínguez et al. 2011; Gui et al. 2017).

Similar studies revealed that biochar-amended soil greatly influenced the diversity and dominant taxa of

soil microbial communities (Kolton et al. 2011; Khodadad et al. 2011). It has been suggested that the ecological shifts in the relative abundance of the taxa can be possibly attributed to several factors. One factor is the enrichment of opportunistic taxa that can metabolize nutrients supplemented by the biochar (increased relative abundance). Other factors may be the toxic effects of biochar on a portion of the microbial community (decreased relative abundance), and changes in the properties of biochar-amended soil (Lozupone and Knight 2008; Khodadad et al. 2011). These also suggest that the mineral heterogeneity in soil contributes to the spatial variation in bacterial communities (Carson et al. 2009), and the nature of changes in microbial community structure depends on the environment (Jenkins et al. 2017). The microbial community structure in the cacao rhizosphere treated with biochar alone has clustered away from the other samples, suggesting the amount and role of organic matter in the soil. Organic matter has the highest contribution in distinguishing the four microbial communities (data not shown), as observed in this study.

The changes in the microbial diversity and composition may also be attributed to the plant-AMF symbiosis (Gui et al. 2017) due to the release of plant rhizodeposits (root exudates) (Vestergård et al. 2008). Certain rhizosphere microorganisms were reported being stimulated (Filion et al. 1999), while others were suppressed by AMF (Wamberg et al. 2003). Biochar and AMF could have contrasting effects on the abundances of rhizobacteria due to the changes in the chemical properties of the acidic cacao rhizosphere.

Besides, this study observed some PGPR, which involved in nitrogen cycling by transforming different forms of nitrogen-containing compounds and supplying the nutrient requirements of the plants (Gruber and Galloway 2008). Cortes et al. (2020) demonstrated the plant-growth promoting attributes (such as nitrogen fixation and phosphate solubilization) of all culturable plant growth-promoting diazotrophic bacteria isolated from the cacao rhizosphere treated with bamboo biochar and AMF, indicating beneficial roles in the growth of *T. cacao*. Together with AMF, these PGPR more than likely helped the cacao plants in nutrient acquisition when grown in nutrient-deficient acidic soil. This synergistic interaction is possible as AMF improved the fine roots dry matter.

CONCLUSION

This study reports the comparative analysis of the microbial diversity in biochar-amended and AMF-inoculated acidic cacao rhizosphere. All treatments reduced the species diversity in the acidic rhizosphere. However, it demonstrated reverse effects on the cacao plant's growth as it was generally improved by the treatments. Although the composition of the microbial communities in the cacao rhizosphere samples is mostly similar, the relative abundances of most of the taxa differ in biochar-amended and AMF-inoculated soil. Also, the AMF activity was stimulated with biochar addition, which generally improved the overall growth of *T. cacao*, AMF root infection, and soil chemical properties. The stimulation of important plant growth-promoting microorganisms and plant growth through the application of AMF and bamboo biochar pose important indications in agricultural development, which benefits the farmers and other cacao-related industries in adopting an alternative agricultural option to gain better cacao production.

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REFERENCES CITED

AGGANGAN NS, CORTES AD, OPULENCIA RB, JOMAO-AS JG, YECYEC RP. 2019a. Effects of mycorrhizal fungi and bamboo biochar on the rhizosphere bacterial population and nutrient uptake of cacao (*Theobroma cacao* L.) seedlings. Phil J Crop Sci 44 (1): 1-9.

AGGANGAN NS, CORTES AD, REAÑO CE. 2019b. Growth response of cacao (*Theobroma cacao* L.) plant as affected by bamboo biochar and arbuscular mycorrhizal fungi in sterilized and unsterilized soil. Biocatal Agric Biotechnol 101347 p.

AISLABIE J, DESLIPPE JR. 2013. Soil microbes and their contribution to soil services. In: Dymond, JR (ed.) Ecosystem Services in New Zealand – Conditions and trends. Manaaki Whenua Press, Lincoln, New Zealand, 143-161.

AKYOL TY, NIWA R, HIRAKAWA H, MARUYAMA H, SATO T, SUZUKI T, FUKUNAGA A, SATO T, YOSHIDA S, TAWARAYA K, SAITO M. 2019. Impact of introduction of arbuscular mycorrhizal fungi on the root microbial community in agricultural fields. Microbes Environ 34: 23-32.

ANANDAN R, DHARUMADURAI D, MANOGARAN GP. 2016. An introduction to actinobacteria. In: Dharumadurai, D., Yi, J. (eds.) Actinobacteria-Basics and Biotechnological Applications. IntechOpen.

ANDERSON CR, CONDRON LM, CLOUGH TJ, FIERS M, STEWART A, HILL RA, SHERLOCK RR. 2011. Biochar induced soil microbial community change: implications for biogeochemical cycling of carbon, nitrogen and phosphorus. Pedobiologia 54 (5-6): 309-320.

ARGOUT X et al. 2011. The genome of *Theobroma cacao*. Nat Genet 43(2): 101-108.

AZCÓN-AGUILAR C, BAREA JM. 2015. Nutrient cycling in the mycorrhizosphere. J Plant Nutr Soil Sci 15: 372-396.

BARKA EA, VATSA P, SANCHEZ L, GAVEAU-VAILLANT N, JACQUARD C, KLENK HP, CLÉMENT C, OUHDOUCH Y, VAN WEZEL GP. 2016. Taxonomy, physiology, and natural products of Actinobacteria. Microbiol Mol Biol Rev 80 (1): 1-43.

BARONTI S, ALBERTI G, DELLE VEDOVE G, DI GENNARO F, FELLET G, GENESIO L, MIGLIETTA F, PERESSOTTI A, VACCARI, FP. 2010. The biochar option to improve plant yields: first results from some field and pot experiments in Italy. Ital J Agron 5: 3-12.

BIEDERMAN LA, HARPOLE WS. 2013. Biochar and its effects on plant productivity and nutrient cycling: a meta-analysis. Glob Change Biol Bioenergy 5: 202-214.

BULGARELLI D, SCHLAEPPI K, SPAEPEN S, VAN THEMAAT EVL, SCHULZE-LEFERT P. 2013. Structure and functions of the bacterial microbiota of plants. Annu Rev Plant Biol 64: 807-838.

CARSON JK, CAMPBELL L, ROONEY D, CLIPSON N, GLEESON DB. 2009. Minerals in soil select distinct bacterial communities in their microhabitats. FEMS Microbiol Ecol 67(3): 381-388.

CHEN J, LIU X, ZHENG J, ZHANG B, LU H, CHI Z, PAN G, LI L, ZHENG J, ZHANG X, WANG J, YU X. 2013. Biochar soil amendment increased bacterial but decreased fungal gene abundance with shifts in community structure in a slightly acid rice paddy from Southwest China. Appl Soil Ecol 71: 33-44.

COMPANT S, DUFFY B, NOWAK J, CLÉMENT C, BARKA EA. 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 71 (9): 4951-4959.

CORTES AD, OPULENCIA RB, AGGANGAN NS. 2020. Characterization of plant growth promoting diazotrophic bacteria isolated from cacao (*Theobroma cacao* L.) Rhizosphere treated with bamboo biochar and arbuscular mycorrhizal fungi. Philip J Sci 149 (4): 1063 -1070.

DEPARTMENT OF TRADE AND INDUSTRY. 2017. In: The Philippines in the cocoa-chocolate global value chain. Policy briefs series no. 2017-09. Retrieved March 27, 2020 from http://industry.gov.ph/wp-content/uploads/2017/11/DTI-Policy-Brief-2017-09-The-Philippines-in-the-CocoaChocolate-Global-Value-Chain.pdf.

DUCEY TF, NOVAK JM, JOHNSON MG. 2015. Effects of biochar blends on microbial community composition in two coastal plain soils. Agriculture 5: 1060-1075.

DUNFIELD PF, YURYEV A, SENIN P, SMIRNOVA AV, STOTT MB, HOU S, LY B, SAW JH, ZHOU Z, REN Y, WANG J. 2007. Methane oxidation by an extremely acidophilic bacterium of the phylum *Verrucomicrobia*. Nature 450: 879-882.

ELAD Y, CYTRYN E, HARREL YM, LEW B, GRABER ER. 2011. The biochar effect: plant resistance to biotic stresses. Phyophatol Mediterr 50: 335-349.

FERNÁNDEZ-GÓMEZ B, MALDONADO J, MANDAKOVIC D, GAETE A, GUTIÉRREZ RA, MAASS A, CAMBIAZO V, GONZÁLEZ M. 2019. Bacterial communities associated to Chilean altiplanic native plants from the Andean grasslands soils. Sci Rep 9: 1042 p.

FILION, M, ST-ARNAUD M, FORTIN JA. 1999. Direct interaction between the arbuscular mycorrhizal fungus *Glomus* intraradices and different rhizosphere microorganisms. New Phytol 141 (3): 525-533.

GAO L, WAN R, SHEN G, ZHANG J, MENG G, ZHANG J. 2017. Effects of biochar on nutrients and

the microbial community structure of tobacco-planting soils. J Soil Sci Plant Nutr 17(4): 884-896.

GKARMIRI K, MAHMOOD S, EKBLAD A, ALSTRÖM S, HÖGBERG N, FINLAY R. 2017. Identifying the active microbiome associated with roots and rhizosphere soil of oilseed rape. Appl Environ Microbiol 83 (22): e01938-17.

GOTELLI NJ, COLWELL RK. 2011. Estimating species richness. Biological diversity: frontiers in measurement and assessment 12: 39-54.

GRABER ER, ELAD Y. 2013. Biochar impact on plant resistance to disease. In: Ladygina N, Rineau F (eds) Biochar and soil biota. CRC Press, Boca Raton. p. 41-68

GRUBER N, GALLOWAY JN. 2008. An earth-system perspective of the global nitrogen cycle. Nature 451: 293-296.

GUI H, PURAHONG W, HYDE KD, XU J, MORTIMER PE. 2017. The arbuscular mycorrhizal fungus *Funneliformis mosseae* alters bacterial communities in subtropical forest soils during litter decomposition. Front Microbiol 8: 1120.

HARTMANN A, SCHMID M, VAN TUINEN D, BERG G. 2009. Plant-driven selection of microbes. Plant Soil 321: 235-257.

HUG LA, MAPHOSA F, LEYS D, LÖFFLER FE, SMIDT H, EDWARDS EA, ADRIAN L. 2013. Overview of organohalide-respiring bacteria and a proposal for a classification system for reductive dehalogenases. Philosophical Transactions of the Royal Society B: Biological Sciences 368(1616): p. 20120322.

IGIEHON NO, BABALOLA OO. 2018. Rhizosphere microbiome modulators: contributions of nitrogen fixing bacteria towards sustainable agriculture. Int. J. Environ. Res. Public Health 15(4): 574.

IMHOFF JF. 2014. The family *Chromatiaceae*, In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F (eds) The Prokaryotes. Springer, Berlin, Heidelberg. p. 151-178

ISHII T, KADOYA K. 1994. Effects of charcoal as a soil conditioner on citrus growth and versicular-arbuscular mycorrhizal development. J Jpn Soc Hortic Sci 63: 529-535.

JENKINS JR, VIGER M, ARNOLD EC, HARRIS ZM, VENTURA M, MIGLIETTA F, GIRARDIN C, EDWARDS RJ, RUMPEL C, FORNASIER F, ZAVALLONI C. 2017. Biochar alters the soil microbiome and soil function: results of next-generation amplicon sequencing across Europe. Glob Change Biol Bioenergy 9 (3): 591-612.

KASSAMBARA A, MUNDT F. 2019. Factoextra: extract and visualize the results of multivariate data analysis. R package version 1.0.6. Retrieved March 25, 2020. from https://CRAN.R-project.org/package=factoextra.

KHODADAD CLM, ZIMMERMAN AR, GREEN SJ, UTHANDI S, FOSTER JS. 2011. Taxa-specific changes in soil microbial community composition induced by pyrogenic carbon amendments. Soil biol biochem 43: 385-392.

KOLTON M, HAREL YM, PASTERNAK Z, GRABER ER, ELAD Y, CYTRYN, E. 2011. Impact of biochar application to soil on the root-associated bacterial community structure of fully developed greenhouse pepper plants. Appl environ microbiol 7: 4924-4930.

KOLTON M, GRABER ER, TSEHANSKY L, ELAD Y, CYTRYN E. 2017. Biochar-stimulated plant performance is strongly linked to microbial diversity and metabolic potential in the rhizosphere. New Phytol 213: 1393-1404.

KÖBERL M, DITA M, MARTINUZ A, STAVER C, BERG G. 2017. Members of *Gammaproteobacteria* as indicator species of healthy banana plants on Fusarium wilt-infested fields in Central America. Sci Rep 7: 45318.

LE S, JOSSE J, HUSSON F. 2008. FactoMineR: An R package for multivariate analysis. J Stat Soft 25 (1): 1-18.

LEHMANN J, JOSEPH S. (eds.) 2015. Biochar for environmental management: science, technology and implementation. Routledge.

LEHMANN J, RILIG MC, THIES J, MASIELLO CA, HOCKADAY WC, CROWLEY D. 2011. Biochar effects on soil biota – A review. Soil biol biochem 43: 1812-1836.

LINDSTRÖM K, ASERSE AA, MOUSAVI SA. 2015. Evolution and taxonomy of nitrogen-fixing organisms with emphasis on rhizobia. Biological nitrogen fixation 1: 21-38.

LIU X, ZHANG S, JIANG Q, BAI Y, SHEN G, LI S, DING W. 2016. Using community analysis to explore bacterial indicators for disease suppression of tobacco bacterial wilt. Sci Rep 6: 36773.

LLADÓ S, LÓPEZ-MONDÉJAR R, BALDRIAN P. 2017. Forest soil bacteria: diversity, involvement in ecosystem processes, and response to global change. Microbiol Mol Biol Rev 81 (2): e00063-16.

LOZUPONE CA, KNIGHT R. 2008. Species divergence and the measurement of microbial diversity. FEMS Microbiol Rev 32 (4): 557-578.

LU Y, RAO S, HUANG F, CAI Y, WANG G, CAI K. 2016. Effects of biochar amendment on tomato bacterial wilt resistance and soil microbial amount and activity. Int J Agron Article ID 2938282. 10 p.

MAHESHWARI DK, DHEEMAN S, AGARWAL M. 2015. Phytohormone-producing PGPR for sustainable agriculture, In: Maheshwari D (eds) Bacterial metabolites in sustainable agroecosystem. Sustainable development and biodiversity. Springer, Cham. p. 159-182.

MALTZ MR, CHEN Z, CAO J, AROGYASWAMY K, SHULMAN H, ARONSON EL. 2019. Inoculation with *Pisolithus tinctorius* may ameliorate acid rain impacts on soil microbial communities associated with *Pinus massoniana* seedlings. Fungal Ecol 40: 50-61.

MEENA M, SWAPNIL P, ZEHRA A, AAMIR M, DUBEY MK, GOUTAM J, UPADHYAY RS. 2017. Beneficial microbes for disease suppression and plant growth promotion, In: Singh D, Singh H, Prabha R, editors. Plant-Microbe Interactions in Agro-Ecological Perspectives. Springer, Singapore. p. 395-432.

MEHTA P, WALIA A, SHIRKOT CK. 2015. Functional diversity of phosphate solubilizing plant growth promoting rhizobacteria isolated from apple trees in the trans Himalayan region of Himachal Pradesh, India. Biol Agric Hortic 31 (4): 265-288.

MORGAN JAW, WHIPPS JM. 2001. Methodological approaches to the study of rhizosphere carbon flow and microbial population dynamics. In: Pinton A, Varanini Z, Nannipieri P, editors. The rhizosphere. Biochemistry and organic substances at the soil-plant interface. Marcel Dekker, New York, USA. p. 373-409.

NAETHER A, FOESEL BU, NAEGELE V, WÜST PK, WEINERT J, BONKOWSKI M, ALT F, OELMANN Y, POLLE A, LOHAUS G, GOCKEL S. 2012. Environmental factors affect acidobacterial communities below the subgroup level in grassland and forest soils. Appl Environ Microbiol 78: 7398-7406.

PINI F, GALARDINI M, BAZZICALUPO M, MENGONI A. 2011. Plant-bacteria association and symbiosis: are there common genomic traits in Alphaproteobacteria?. Genes 2: 1017-1032.

QUAST C, PRUESSE E, YILMAZ P, GERKEN J, SCHWEER T, YARZA P, PEPLIES J, GLÖCKNER FO. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids res 41: 590-596.

QUILLOY KP. 2015. Empowering small farmers through cooperative: the success story of Subasta integrated farmers multi-purpose cooperative. IRMBR 4(1): 361-375.

R CORE TEAM. 2018. R: A language and environment for statistical computing R foundation for statistical computing. Vienna, Austria Retrieved March 25, 2020 from https://www.R-project.org/.

RADHAKRISHNAN R, HASHEM A, ABD_ALLAH EF. 2017. Bacillus: a biological tool for crop improvement through bio-molecular changes in adverse environments. Front Physiol 8: 667.

ROGER A, COLARD A, ANGELARD C, SANDERS IR. 2013. Relatedness among arbuscular mycorrhizal fungi drives plant growth and intraspecific fungal coexistence. ISME J 7: 2137-2146.

ROUSK J, BÅÅTH E, BROOKES PC, LAUBER CL, LOZUPONE C, CAPORASO JG, KNIGHT R, FIERER N. 2010. Soil bacterial and fungal communities across a *pH* gradient in an arable soil. ISME J 4: 1340-51.

SANTANA RS, FERNANDES GW, ÁVILA MP, REIS MP, DE ARAÚJO FM, SALIM A, OLIVEIRA G, CHARTONE-SOUZA E, NASCIMENTO A. 2016. Endophytic microbiota associated with the root tips and leaves of *Baccharis dracunculifolia*. Braz Arch Biol Technol 59: e16160287.

SAYYED RZ, CHINCHOLKAR SB, REDDY MS, GANGURDE NS, PATEL PR. 2013. Siderophore producing PGPR for crop nutrition and phytopathogen suppression, In: Maheshwari D (eds) Bacteria in agrobiology: Disease management. Springer, Berlin, Heidelberg. p.449-471.

SCHREITER S, DING GC, HEUER H, NEUMANN G, SANDMANN M, GROSCH R, KROPF S, SMALLA K. 2014. Effect of the soil type on the microbiome in the rhizosphere of field-grown lettuce. Front Microbiol 5 (144): 1-13.

SHANNON CE. 1948. A mathematical theory of communication. Bell Syst The J. Blackwell Publishing Ltd; 27: 379-423.

SIMPSON EH. 1949. Measurement of diversity. Nature 163: 688-688.

SMITH SE, JAKOBSEN I, GRØNLUND M, SMITH FA. 2011. Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. Plant Physiol 156: 1050-1057.

SMITH SE, READ DJ. 2010. Mycorrhizal symbiosis. Academic press. p. 113.

SOLÍS-DOMÍNGUEZ FA, VALENTÍN-VARGAS A, CHOROVER J, MAIER RM. 2011. Effect of arbuscular mycorrhizal fungi on plant biomass and the rhizosphere microbial community structure of mesquite grown in acidic lead/zinc mine tailings. Sci total environ 409 (6): 1009-16.

SULLIA SB. 1991. Use of vesicular-arbuscular mycorrhiza (VAM) as biofertilizer for horticultural plants in developing countries. In: Prakash J, Pierik RLM (eds.) Horticulture—New technologies and applications. Dordrecht: Springer. p. 49-53.

TAKADA-HOSHINO Y, MATSUMOTO N. 2004. An improved DNA extraction method using skim milk from soils that strongly adsorb DNA. Microbes environ 19 (1): 13-19.

VESTERGÅRD M, HENRY F, RANGEL-CASTRO JI, MICHELSEN A, PROSSER JI, CHRISTENSEN S. 2008. Rhizosphere bacterial community composition responds to arbuscular mycorrhiza, but not to reductions in microbial activity induced by foliar cutting. FEMS Microbiol Ecol 64: 78-89.

WAMBERG C, CHRISTENSEN S, JAKOBSEN I, MÜLLER AK, SØRENSEN SJ. 2003. The mycorrhizal fungus (*Glomus intraradices*) affects microbial activity in the rhizosphere of pea plants (*Pisum sativum*). Soil Biol Biochem 35 (10): 1349-1357.

WANG F. 2017. Occurrence of arbuscular mycorrhizal fungi in mining-impacted sites and their contribution to ecological restoration: mechanisms and applications. Crit Rev Environ Sci Technol 47 (20): 1901-1957.

WARNOCK DD, LEHMANN J, KUYPER TW, RILLIG MC. 2007. Mycorrhizal responses to biochar in soil–concepts and mechanisms. Plant Soil 300: 9-20.

WEI L, OUYANG S, WANG Y, SHEN X, ZHANG L. 2014. *Solirubrobacter phytolaccae* sp. nov., an endophytic bacterium isolated from roots of *Phytolacca acinosa* Roxb. Int J Syst Evol Microbiol 64(3): 858-862.

WICKHAM H. 2016. ggplot2: Elegant graphics for data analysis. Springer-Verlag New York.

WINSLEY TJ, SNAPE I, MCKINLAY J, STARK J, VAN DORST JM, JI M, FERRARI BC, SICILIANO SD. 2014. The ecological controls on the prevalence of candidate division TM7 in polar regions. Front Microbiol 5: 345.

WOOLF D, AMONETTE JE, STREET-PERROTT FA, LEHMANN J, JOSEPH S. 2010. Sustainable biochar to mitigate global climate change. Nat Commun 1: 56.

XU N, TAN G, WANG H, GAI X. 2016. Effect of biochar additions to soil in nitrogen leaching, microbial biomass and bacteria community structure. Eur. J. Soil Biol. 74: 1-8.

YANG SJ, ZHANG ZL, XUE YX, ZHANG ZF, SHI SY. 2014. Arbuscular mycorrhizal fungi increase salt tolerance of apple seedlings. Bot Stud 55: 1-7.

YU Y, LEE C, KIM J, HWANG S. 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol Bioeng 89: 670-679.