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Growth response of cacao (*Theobroma cacao* L.) plant as affected by bamboo biochar and arbuscular mycorrhizal fungi in sterilized and unsterilized soil

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ABSTRACT

Biochar and arbuscular mycorrhizal fungi (AMF) are found to improve crop productivity affecting the nutrient availability in the soil and favoring build-up of beneficial microbes. The effects of AMF and bamboo biochar (BB) on the growth of cacao, estimated number of mycorrhizal spores, and soil chemical properties were investigated. Cacao seedlings were grown in unsterilized and oven-sterilized acidic soil. After 15 months, AMF generally increased most growth traits of cacao plant over the control. Bamboo biochar at 15%, gave the best plant growth regardless of soil sterilization. When biochar was added to AMF, 15% consistently provided the heaviest total plant dry weight, especially in unsterilized soil. Likewise, all treatments improved the height and stem diameter increments of those grown in unsterilized soil. Percentage of mycorrhizal root colonization was higher in mycorrhizal (+AMF) plant and addition of biochar proliferated AMF spores in the rhizosphere. Biochar showed positive effect in enhancing the soil chemical properties. The results imply that biochar and AMF can improve the overall growth and can positively increase the yield of cacao plants, which will provide impact in improving cacao production in acidic soils in the Philippines.

1. Introduction

The global demand of cacao (*Theobroma cacao* L.) is expected to reach between 4.7 and 5 million metric tons (MT) by the year 2020 (Bureau of Plant Industry, 2017) and the global supply will be at a deficit of 1 million MT. The consumption in the Philippines is 50,000 MT annually, but the local supply is only around 10,000 MT, making the country a net importer (Cacao Industry Development Association of Mindanao Inc., 2017). Thus, with the growing demand for cocoa products, the world may soon run out of chocolate. The Philippine Cacao Challenge commits to produce 100,000 MT by the year 2020 and onwards (Bureau of Plant Industry, 2017).

There is a need to do interventions, such as application of biofertilizer and soil amendment, to facilitate plant growth and increase bean yield and survival. These interventions should aim to produce organically grown cocoa beans for health benefits.

Arbuscular mycorrhizal fungi (AMF) can improve plant growth and increase yield especially in nutrient deficient acidic soil and as a substitute to chemical fertilizer and other farm inputs. These fungi are natural biofertilizers that provide plants enough nutrients while establishing protection against pathogens in exchange for plant photosynthetic products (Wang et al., 2017; Berruti et al., 2016). AMF form a mutual symbiotic relationship with about 80% of land plants through root colonization (Hildebrandt et al., 2002; Berruti et al., 2016). The mutual symbiotic effectiveness varies among individual plant-fungus combinations (Smith et al., 2003) and the success of this relationship is unpredictable (Berruti et al., 2016). Generally, arbuscular mycorrhizal symbiosis helps the plant attain its optimal growth level because of the beneficial nutrient acquisition activity performed by AMF (Wang et al., 2017). The use of AMF is promising for an environment-friendly agriculture, which could reduce the use of chemical fertilizers and pesticides yet still improve the yield of various crops (Kim et al., 2017).

Biochar is a thermally decomposed carbon-rich product obtained through pyrolysis that establishes a significant, long-term sink for atmospheric carbon dioxide by reducing emissions and increasing sequestration of screenhouse gases in terrestrial ecosystems (Lehmann et al., 2006). Its beneficial effects include improvement of soil fertility and promotion of nutrient cycling (Lehmann et al., 2006; Biederman and Harpole, 2013). It can serve as a refuge for colonizing fungi and bacterial communities, giving them protection against natural soil predators

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(Warnock et al., 2007). In addition, application of biochar to acidic soil results in good soil chemical and biological environment, which positively influenced the growth and yield of the crop (Pandian et al., 2016). Biochar benefits agriculture and farming systems in low-nutrient, acidic soils, especially in tropical countries (Jeffery et al., 2017).

Biochar has been reported to have mean residence in soil up to 10,000 years (Skjemstad et al., 1998; Swift, 2001), indicating its long-term benefits especially in acidic soils. Sources of producing biochar have positive effects on plant growth. In Indonesia, Cornelissen et al. (2018) reported that biochar derived from cacao shells improved the growth of maize crop grown in ultisol (pH of 3.6) due to the soil acidity alleviation. Reapplication of biochar was necessary for rice hull and cacao shell after 3 to 5 seasons, respectively (Cornelissen et al., 2018). In Thailand and Philippines, four-season field trials were conducted using rice husk biochar, which reported an increase in yield by 16–35% due to the improvements in water retention and increased available K and P (Haefele et al., 2011). Similar studies also observed the positive effect of biochar in acidic soils for multiple planting seasons (Steiner et al., 2007; Major et al., 2010).

In the Philippines, research on biochar as soil amendment to increase soil fertility of acidic soil has been recently explored. It was observed that the effect of this biochar in the soil and growth of plants may vary depending on the type of biomass sources. Bamboo trimmings from bamboo-related industries are normally burned to avoid pile up of unwanted wastes. However, bamboo is hard to decompose if left unattended, thus, converting these bamboo wastes into biochar and be used as soil amendment in acidic soil is an innovative technology for farmers. Nevertheless, the benefits should be demonstrated prior to adaption by farmers.

Combined application of biochar and AMF can further improve plant growth and soil health status (Lehmann et al., 2011). Improved yield of various plants has been reported on wheat (Solaiman et al., 2010), pelargonium (Conversa et al., 2015), lettuce (Hammer et al., 2015), and sorghum (Ortas, 2016). Although the use of biochar and AMF has recently been explored to improve crop production, this is the first study that uses biochar from bamboo in combination with mycorrhizal fungi applied on cacao. Cacao is a long-term crop, biochar as reported can support increasing crop yield until three to five seasons (depending on biochar source) without reapplication (Cornelissen et al., 2018) while mycorrhizal fungi proliferate with time in the roots of host plants especially in acidic soil. Cacao also forms association with AMF communities (Cuenca and Meneses, 1996) and selected species of these AMF significantly improved the overall growth of cacao seedlings (Cuenca et al., 1990; Tchameni et al., 2011).

The presence of ubiquitous soil-inhabiting microorganisms can, for example, greatly affect mycorrhizal formation. Some studies have reported that soil microbiota enhance the germination of AM fungal spores, the level of root colonization by AM fungi and mycorrhizal plant growth (Azcon-Aguilar et al., 1986; Azcon et al., 1990; Meyer and Linderman, 1986). Molecular analysis demonstrated the bacterial communities attached to AMF hyphae, suggesting their involvement in mycorrhizal interactions (Scheublin et al., 2010). However, there are contradictory evidences that soil microbiota suppress plant growth, mycorrhizal root colonization, sporulation and mycorrhizal fungal spore germination (Hetrick et al., 1988; Wilson et al., 1988).

In a greenhouse experiment, soil sterilization methods (gamma radiation and aerated steam) influenced arbuscular mycorrhizal colonization, nutrition and growth of peanuts plants (*Arachis hypogea* cv. Virginia Bunch) (Middleton et al., 1989). Middleton et al. (1989) also reported that plant dry weight and the number and weight of reproductive structures were reduced to varying extents, depending on how the respective sterilization methods affected subsequent levels of mycorrhizal colonization. Peanuts benefit from mycorrhizal association in sterilized soil, increasing dry matter yield, phosphorus (P) uptake and stimulation of root and shoot growth as a result (Bergero et al., 2003). In another study, Al-Khaliel (2010) studied the effects of arbuscular mycorrhization in sterile and non-sterile soils and reported that *Glomus mosseae* and *Glomus fasciculatum* were infective to peanut but displayed a differential impact on peanut growth depending on the microbial biomass content of the substrate soils. On forest tree species, Aggangan et al. (1996) reported a significant interaction between inoculation, soil fumigation and phosphorus supply on mycorrhizal formation by an ectomycorrhizal fungus *Pisolithus* from Australian and from the Philippines on *Eucalyptus urophylla*. They also reported that soil fumigation enhanced mycorrhizal formation by the Australian *Pisolithus* but did not affect root colonization by the Philippine isolate. Production of nursery raised planting materials should follow protocols that favor root colonization by the preferred mycorrhizal fungi. Predicting the effectiveness of the chosen mycorrhizal fungi in the field, and comparison with previous screenhouse or glasshouse experiments, requires preliminary testing of the fungi in the presence of indigenous soil microflora (Aggangan et al., 1996).

This screenhouse study assessed the effects of bamboo biochar amendment at different level and AMF inoculation on soil chemical properties, estimated count of mycorrhizal spores and cacao plant grown in oven sterilized or unsterilized soil. The results will dictate nurserymen to produce cacao planting materials in growing medium (whether sterile or not sterile) that support mycorrhizal proliferation before field planting in a very acidic to slightly acidic areas intended for cacao production in the Philippines. It is highly recommended that cacao planting materials should be fully colonized with compatible mycorrhizal fungi before field planting.

2. Materials and methods

2.1. Experimental design

Two concurrent (using oven sterilized or unsterilized soil) experiments were conducted following a two factor in Randomized Complete Block Design with 7 replicates for height and stem diameter growth parameters while the other parameters have used three replicates. The substrate mixtures were as follows (in g): soil:biochar 2000:0, soil:biochar 1850:150 (v:v), soil:biochar 1700:300 (v:v) indicated as 0, 7.5, and 15% BB, respectively; and AMF inoculation (+AMF) or without AMF (-AMF).

The experiment was conducted in a screenhouse of the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB), Laguna, Philippines. The screenhouse is enclosed with welded wire lined with white fine nets (to prevent entry of insects), with plastic roofing and sunlight penetration is 90–100%.

2.2. Soil collection, preparation and physico-chemical characteristics

The soil used was collected (0–15 cm depth) during summer, in the Caliraya-Lumot-Cavinti Watershed area, in Cavinti, Laguna, Philippines. According to locals, the collection site was previously covered with dipterocarp (top priced wood in the country) forests many decades ago that became barren for over a decade due to illegal logging. At present, the area is covered with patches of creepers such as carabao grasses.

The soil was brought to the mycorrhiza laboratory of BIOTECH, UPLB, air-dried, and passed through a 5-mm screen and subjected for analyses. The soil has about 27–30% moisture content and water holding capacity of 1.8 mm cm^{-1} depth of soil. Prior to sterilization, the soil contains 2 spores of AMF (*Glomus*) per 10 g of soil.

The soil is bright red color and clay loam texture with acidic pH (4.1 in H_2O). The chemical analyses are the following: 1.12% OM content, 0.03% N (Black, 1965), and 1.3 mg kg^{-1} available P. The exchangeable cations (meq 100 g $soil^{-1}$) are: 0.14 K, 3.26 Ca, 0.09 Mg, and 9.9 CEC. The soil also contains 62.4 mg kg^{-1} Al, 17 mg kg^{-1} Fe and 1 mg kg^{-1} Zn. Chemical analyses were done at the Central Analytical Service Laboratory (CASL) of BIOTECH, UPLB.

2.3. Preparation of biochar

Bamboo wastes from bamboo industries in Calamba, Laguna, Philippines were collected, sun dried, chopped and pyrolyzed at 500 °C inside a fabricated close tank under limited oxygen supply. The closed tank was fabricated at the Forest Products Research and Development Institute, Department of Science and Technology, Los Baños, Laguna. After an overnight cooling, the pyrolyzed bamboo wastes were soaked for 24 h, air-dried and then pulverized in a stainless mill crushing and mixing machine installed at the Pilot Plant of BIOTECH, UPLB.

2.4. Experimental setup

The powdered biochar was cured for two weeks prior to use in the experimentation. Bamboo biochar at different level (0, 7.5, 15% (w/w) was mixed with oven-sterilized (100 °C for 72 h) and unsterilized acidic soil collected from marginal areas in Caliraya, Laguna, Philippines. A final weight of two kg substrate mixtures was placed into each individual expanded (4″x10″x2″) black polyethylene bag, watered to field capacity and incubated for another two weeks before cacao seedlings were planted.

Cacao (UF18) seeds were obtained from cacao growers in Davao, Philippines through Option Inc. (Sto. Tomas, Batangas). Upon arrival at BIOTECH UPLB, the seeds (with radicle) were sown immediately in oven-sterilized (100 °C for 72 h) garden soil-sand (1:1 v/v) mixture and grown inside the nursery. After two weeks, the seedlings were transferred into individual black polyethylene bags filled with different substrate mixtures that were previously cured for two weeks.

2.5. Mycorrhizal inoculation

The AMF inoculant was prepared as described by Aggangan and Moon (2013). Surface sterilized spores were mass produced for six months in beds filled with oven sterilized sand-soil (1:1 v/v) mixture planted to bahia grass as host plant under screenhouse conditions. The soil-based powder AMF inoculant contained a mixture of 12 species belonging to genera *Glomus, Gigaspora, Entrophospora,* and *Acaulospora.* This mycorrhizal inoculant was developed and commercially produced at BIOTECH UPLB, Philippines. The inoculant contained spores (100–150 spores per gram soil inoculant), chopped mycorrhiza infected roots (85–100% colonization), and other infective propagules. The seedlings were inoculated with AMF at the rate of 5 g per seedling placed in a 2–3 inches deep hole beneath and in contact with the roots. Uninoculated non-mycorrhizal treated seedlings were inoculated with 5 g sterilized (20 psi for 15 min) AMF inoculant. This was done to minimize the effect of other factors present in the soil sand carrier of the mycorrhizal inoculant.

2.6. Watering and maintenance

The screenhouse has an average maximum and minimum temperatures of 30 ± 5 and 25 ± 5 °C, respectively. Watering was done by weight (field capacity) and polybags were rearranged once a week in order to minimize the error due to non-homogenous sunlight penetrating inside the screenhouse. Experimental seedlings were placed on steel (1 m width and 5 m length) benches half meter off the ground, rearranged once a week to minimize any non-homogenous sunlight penetrating inside the screenhouse. One g of NPK fertilizer was applied into each plant once per month, during the first two months after planting.

2.7. Growth parameters measured and sample collection

Height and stem diameter were measured periodically for 15 months at 3-month interval from seven plants per treatment. Increments representing the difference in height or stem diameter of the last and first measurements, were reported in this paper. After 15 months, three plants were harvested. Each polybag was cut into half, to expose the roots and soil of potted cacao. A total of 500 g of soil sample from each treatment were collected where sub samples 490 g and 10 g were subjected to soil chemical analysis and estimation of mycorrhizal spore count, respectively.

The whole plant was cut into parts (root, stem and leaves), washed directly under running water, blot dried in between paper towels, wrapped separately in paper towel, placed in brown paper bags and oven-dried at 60 °C for three days. The dry weight of the roots, stem, and leaves were measured using an electronic weighing scale (Mettler-Toledo GmbH, Laboratory Weighing, Switzerland).

2.8. Nitrogen and phosphorus concentration

The youngest fully expanded leaf (YFEL) from each plant was collected, washed under running water, air-dried, wrapped separately with paper towel, placed inside brown paper bags, oven-dried at 60 °C for 5 days, and ground. Samples were analyzed at the CASL-BIOTECH, UPLB for plant N and P concentrations using AOAC 955.04 and AOAC 931.01 methods, respectively.

2.9. AMF root colonization and number of soil spores

The fine (<0.20 mm diameter) roots were separated from the coarse roots by rubbing the roots manually between palms while submerged in water. The fine roots were collected in a stainless sieve with 2 mm opening and blot dried in between paper towels where 1 g was taken for the assessment of root colonization by AMF. The fine root samples were placed in test tubes filled with 50% ethanol solution.

The roots were cleared and stained with trypan blue following the protocol of Phillips and Hayman (1970). Stained roots were placed in Petri dishes with grid lines observed under a stereomicroscope (Olympus, Japan). All roots that crossed the grid lines were counted and roots with vesicles, hyphae or other AMF propagules were considered as mycorrhiza-infected roots. The percentage of mycorrhizal root colonization was calculated based on the formula used by Ishii and Kadoya (1994). Root colonization by AMF was expressed as the percentage of colonized roots over total roots count multiplied by 100 (Aggangan and Moon, 2013).

The estimated number of spores was counted from a 10 g sub sample from the 500 g rhizosphere sample. Spores were separated from the soil using the wet sieving and centrifugation technique of Brundrett et al. (1996). The estimated spore density was counted based on the method of Moreira et al. (2006) using light microscopy.

2.10. Changes in chemical properties of rhizosphere soil after 15 months

The remaining 480 g sub samples of rhizosphere soil from each treatment were air dried and passed through into stainless steel wire mesh sieve with 2 mm opening prior to analysis. Soil analyses were done at the CASL, BIOTECH, UPLB. Soil pH was measured with a compound electrode into a 1:1 soil water ratio (Black et al., 1965; Jackson, 1958). Soil organic matter (OM) was determined using potassium dichromate oxidation (Walkley, 1947; Jackson, 1958) and total nitrogen (N) by Kjeldahl digestion (Jackson, 1958). Available phosphorus (P) was determined using the Bray method (Bray and Kurtz, 1945) and exchangeable potassium (K) was analyzed using ammonium displacement of exchangeable cations (Peech, 1945). Soil cationic exchange capacity (CEC) was determined by ammonium acetate distillation (Black et al., 1965; Peech, 1945).

2.11. Statistical analyses

Data on periodic height and stem diameter increments were analyzed using repeated measures analysis of variance (MANOVA) of RCBD with seven replicates (SAS version 9.4). Other parameters collected for each

Table 1
Summary ANOVA of the parameters as affected by the main and interaction between arbuscular mycorrhizal fungi inoculation and bamboo biochar amendment on the growth and nitrogen and phosphorus concentration of 15-month-old cacao plants grown in oven sterilized and unsterilized soil.

Source of Variation	Height increment (cm)	Stem diameter increment (cm)	Leaf dry weight (g plant^{-1})	Stem dry weight (g plant^{-1})	Coarse root dry weight (g plant^{-1})	Fine roots dry weight (g plant^{-1})	Total plant dry weight (g plant^{-1})	Nitrogen concentration (mg g^{-1})	Phosphorus concentration (mg g^{-1})
Sterilized soil									
AMF (A)	*	**	**	*	ns	ns	*	ns	ns
Biochar (B)	**	**	***	**	ns	*	***	***	***
A x B	*	***	ns	*	*	**	**	***	*
Unsterilized soil									
AMF (A)	**	*	ns	*	ns	*	*	***	***
Biochar (B)	***	**	*	***	ns	ns	**	***	***
A x B	***	*	*	*	*	ns	**	*	***

ns, *, **, *** indicate not significant, significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$ confidence level, respectively.

Table 2
Mean squares for between subject effects for height and diameter and their corresponding p-values using repeated measures analysis of variance.

Source	DF	Diameter Increment		Height Increment	
		Mean Square	Pr > F	Mean Square	Pr > F
Condition	1	25.89	0.0246	13203.74	<.0001
Inoculum	1	23.97	0.0304	1189.05	0.0484
Condition*Inoculum	1	29.26	0.0172	505.81	0.1945
Treatment	2	176.72	<.0001	2833.38	0.0002
Condition*Treatment	2	38.76	0.0008	1134.65	0.0258
Inoculum*Treatment	2	37.01	0.0011	426.778	0.2421
Condition*Inoculum*Treatment	2	15.71	0.0468	431.12	0.2387
Error	73	4.92		295.074	

Note: Condition: Sterilized or unsterilized soil; Inoculum: Arbuscular mycorrhizal fungi; Treatment: Bamboo biochar levels.

Table 3
Mean squares for within subjects effects and their corresponding p-values adjusted for violation of sphericity assumption.

Source	DF	Height increment		Diameter increment	
		Mean Square	Adj Pr > F H–F-L^{+}	Mean Square	Adj Pr > F H–F-L^{+}
Time	4	66825.43	<.0001	2106.13	<.0001
Time*condition	4	327.52	0.0002	2.46	0.1192
Time*inoculation	4	94.17	0.0824	2.06	0.1748
Time*condition*inoculation	4	67.66	0.1688	7.88	0.0006
Time*treatment	8	138.55	0.0057	11.74	<.0001
Time*condition*treatment	8	59.87	0.1787	3.23	0.0221
Time*inoculum*treatment	8	57.64	0.1955	2.30	0.0926
Time*condition*inoculum*treatment	8	35.56	0.4506	1.58	0.2633
Error (Time)	292	38.01		1.21	

$^{+}$p-values adjusted using Huyn-Feldt-Lecoutre epsilon.

experiment (sterilized or unsterilized soil) were analyzed using ANOVA of two factor in RCBD with three replicates. Treatment means were compared using LSD if ANOVA showed significance at $p < 0.05$. Correlation between total plant dry weight and N and P concentrations was also determined. Statistical analyses were done using MSTATC statistical computer program version 2.10 (MSU, 1989).

3. Results

3.1. General observation

In sterilized soil, a significant interaction between AMF and biochar on all plant growth parameters observed except leaf dry weight. Similarly, there was also a significant interaction between AMF and biochar on all plant growth parameters measured except fine root dry weight of cacao grown in unsterilized soil (Table 1).

3.2. Plant height and stem diameter increments

The sphericity tests indicated that the variance-covariance matrix did not meet the compound symmetry condition for both diameter and height ($p < 0.001$) (data not shown). This clearly violates the independence of errors assumption, characteristic of data from repeated measures design. Univariate analysis of variance with degrees of freedom adjusted using the Huyn-Feldt epsilon showed no significant three- and four-way interaction effects for height increment (Tables 2 and 3).

The differences among treatments varied with time as shown in Fig. 1A–B. The differences between BB treatments and no biochar increased with time implying that the BB enhanced growth in terms of height. The behavior on diameter was quite different with no charcoal and 7.5% BB.

The difference between sterile and non-sterile conditions increased as the months progressed. As shown in Fig. 2A and C, both time x treatment and time x condition, effects seem to diminish starting from month12 to month15 as evidenced by the parallel lines. Between subjects, effects showed significant condition x treatment interaction as

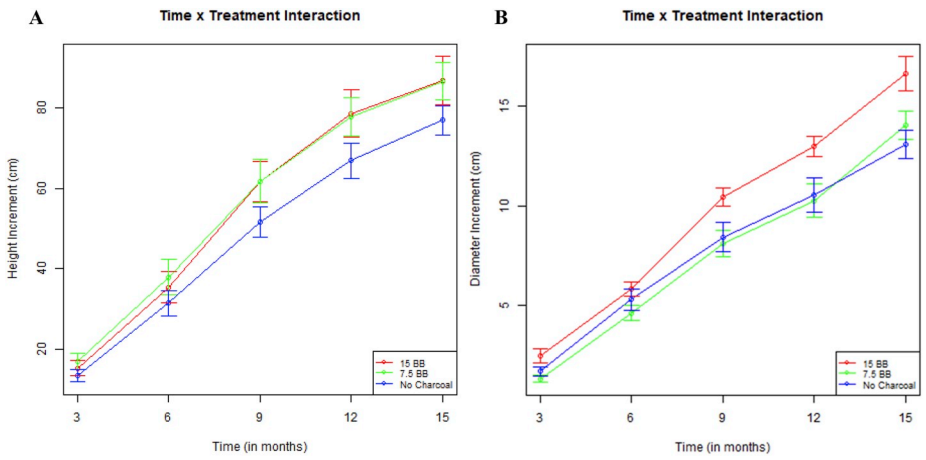

Fig. 1. Periodic height (A) and stem diameter (B) increments of cacao plants grown for 15 months as affected by the interaction of time and treatment (increasing level of biochar from bamboo). Error bars are in 95% confidence interval. N = 7.

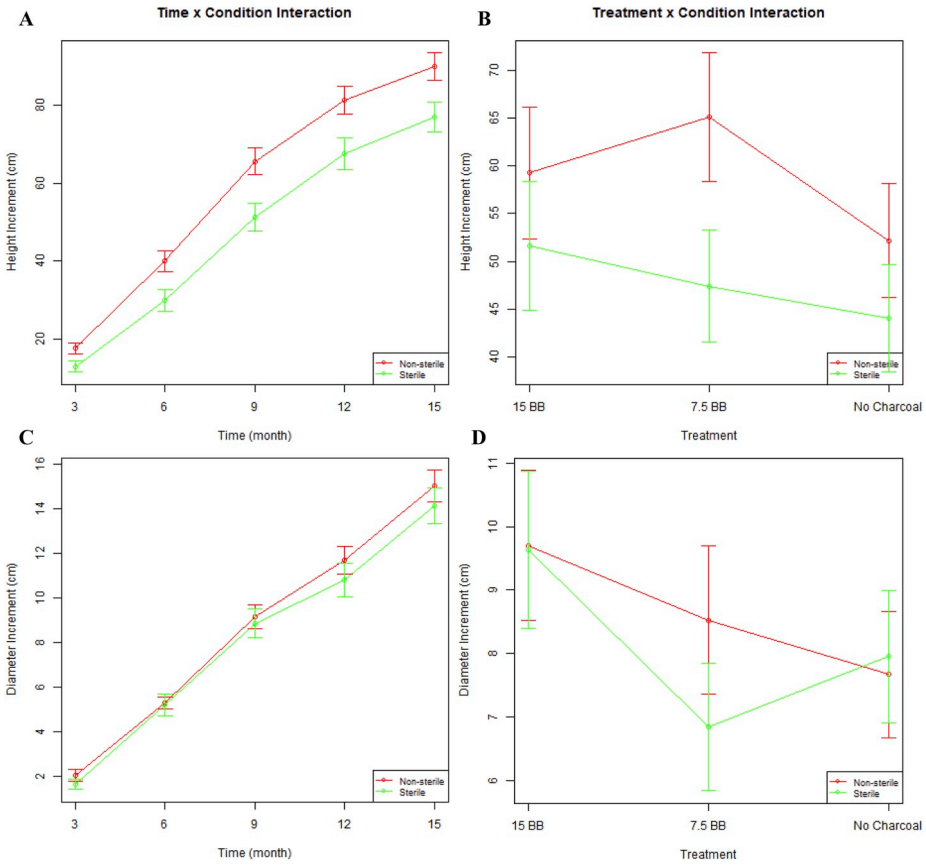

Fig. 2. Periodic height and stem diameter increments of cacao plants grown for 15 months as affected by the interaction of time and condition (A and C) and interaction of treatment and condition (B and D). Error bars are in 95% confidence interval. N = 7.

shown in Fig. 2B and D. Under sterile and non-sterile conditions, height increased with increasing BB levels. However, there was a drastic increase in height at 7.5% BB under non-sterile condition which was detected as significant treatment × condition interaction. Fig. 3 shows that height and stem diameter increase was enhanced with inoculation regardless of time and condition. However, height increase was more pronounced in the 15% BB and in no BB.

Fig. 4 showed the effects of the treatments on the shoot growth during harvest. In sterilized soil, the height (Fig. 4A) and stem diameter (Fig. 4C) of the cacao plants increased when AMF+15% BB was added compared to the control, whereas in unsterilized soil, all treatments increased the height (Fig. 4B) and stem diameter (Fig. 4D) increment values over the control.

3.3. Plant part dry weight

Generally, AMF alone increased the dry weight of the stem, coarse root and fine roots and even the total plant dry matter (Table 4). In sterilized soil, cacao plants inoculated with AMF had increased in dry weight (by 9.5% in leaves, by 16.6% in stem, by 12.2% in coarse root, by

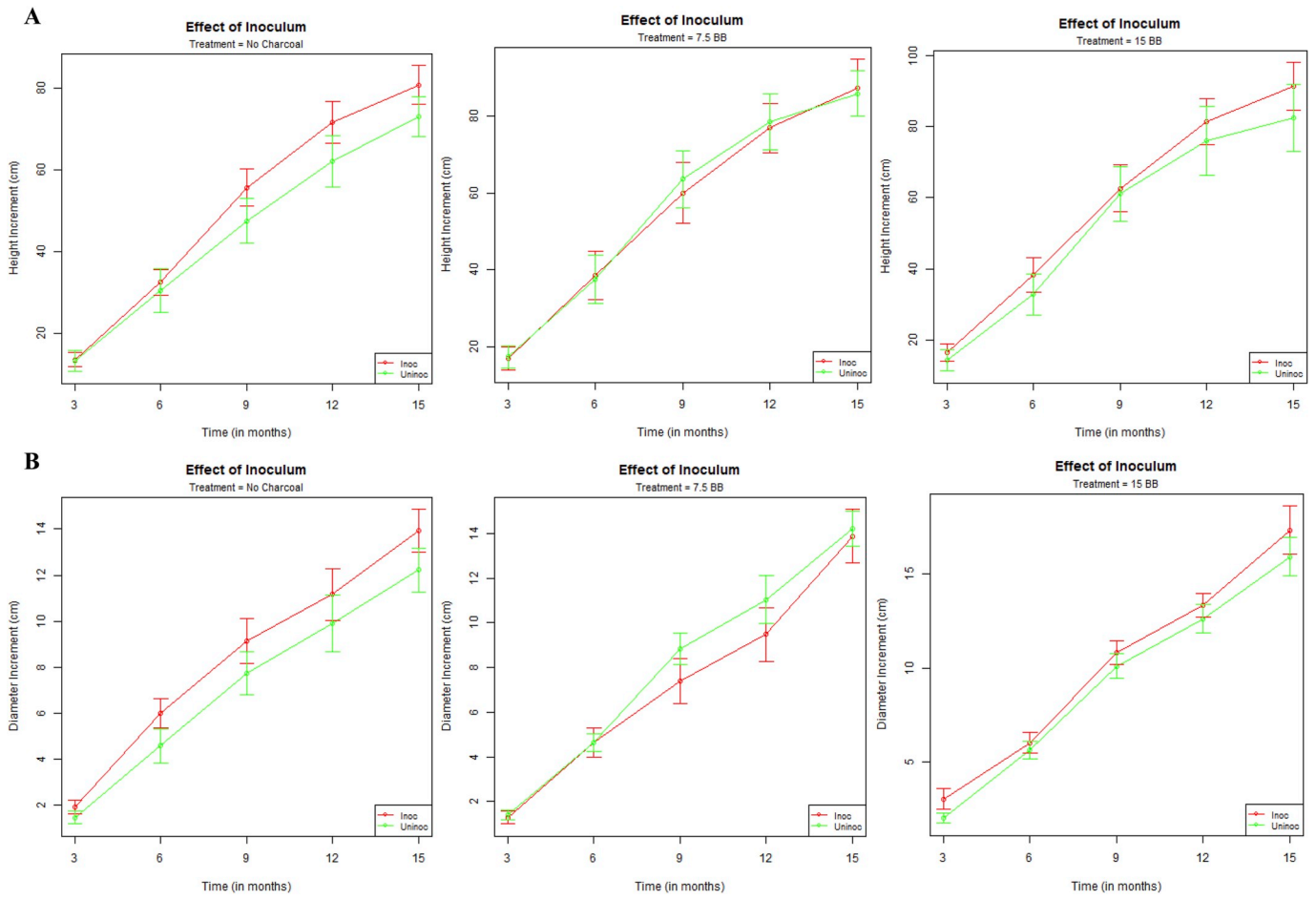

Fig. 3. Effects of AMF inoculation amended with no biochar (A and D), 7.5% BB (B and E), and 15% BB (C and F) on periodic height and stem diameter increment of cacao plants after 15 months. Error bars are in 95% confidence interval. N = 7.

26.5% in fine roots dry weight) relative to non-mycorrhizal (-AMF) counterpart.

In unsterilized soil, AMF increased leaf dry weight by 10.9%, stem dry weight by 4.7%, coarse root dry weight by 12.2%, and fine roots dry weight by 97%. The increase in growth parameters by AMF inoculation was generally higher in the unsterilized soil than in sterilized one (Table 4). Fine roots were the most affected by the interaction between AMF and biochar level. Fine roots were increased by 97% by AMF in unsterilized soil and by 26.5% in sterilized counterpart. In sterilized soil, fine roots of non-mycorrhizal plants were heavier in sterilized soil amended with 15% biochar. By contrast, mycorrhizal cacao plants produced significantly more roots in nil biochar (9.95 g plant^{-1}) than those grown in unsterilized soil amended with 15% biochar (4.22 g plant^{-1}) (Table 4).

Total dry weight was increased by 15.1% when cacao plants were inoculated with AMF and grown in sterilized soil and by 17.8% in unsterilized counterpart (Table 4). Without AMF, the highest total plant dry weight was obtained in plants grown in sterilized soil amended with 15% biochar. The total plant dry weight was comparable in non-mycorrhizal plants grown in unsterilized soil amended with 7.5 and 15% biochar, which was significantly higher than that in the nil biochar (Table 4).

3.4. Nutrient concentration

The N and P concentrations of the YFEL of cacao plants grown in sterilized soil were not significantly affected by the inoculation with AMF (Table 1). By contrast, in unsterilized soil, N and P concentrations were significantly affected with AMF inoculation. There was a significant interaction between biochar and AMF on leaf N and P concentrations of cacao plants grown either in sterilized or unsterilized soils (Table 1).

In the sterilized soil, the highest N and P concentrations were obtained in the non-mycorrhizal plants grown in 7.5% biochar (Table 4). In unsterilized counterpart, the highest (20.35 mg g^{-1}) N concentration was observed in mycorrhizal cacao grown in no biochar while the highest (1.82 mg g^{-1}) P was obtained from non-mycorrhizal plants grown in 15% biochar. Correlation coefficient r^2 between P and total plant dry weight was −0.55, $p < 0.05$ in sterile soil (data not shown). On the other hand, the r^2 between N and total plant dry weight in non-sterile soil was −0.54, $p < 0.05$ (data not shown).

3.5. Mycorrhizal root colonization and spore count

Mycorrhizal inoculation on cacao plants resulted in higher root colonization and spore count (Table 5). Regardless of biochar amendment, AMF inoculation showed increase in root infection by AMF in both sterilized and unsterilized soil. On the other hand, addition of biochar to AMF also improved the estimated number of mycorrhizal spores in the cacao rhizosphere (Table 5). The non-mycorrhizal treated cacao showed no colonization (Fig. 5A), whereas the roots obtained in plants treated with AMF+15% biochar showed the presence of mycelial hyphae (dark blue strands) and the circular-to oblong-shaped vesicles that act as food storage organ of AMF (Fig. 5B).

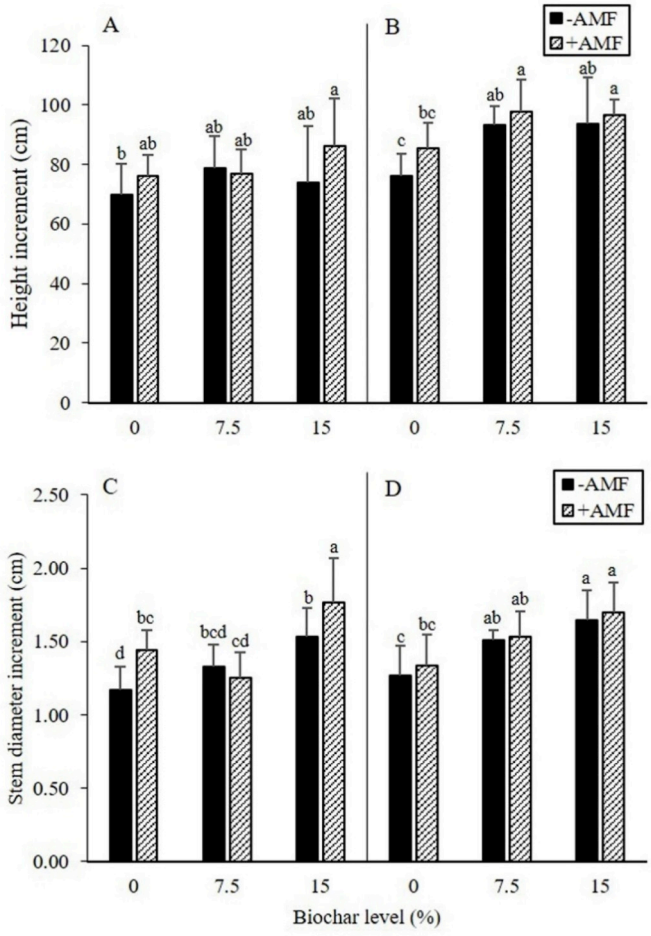

Fig. 4. Height and stem diameter increments of 15-month-old non-mycorrhizal (-AMF) or mycorrhizal (+AMF) cacao plants grown in sterilized soil (A and C) and unsterilized soil (B and D) amended with increasing level (0, 7.5, and 15%) of bamboo biochar. Bars with different letters indicate significant difference using LSD range test at $p < 0.05$. N = 7.

3.6. Soil chemical characteristics

Generally, 15% BB provided the good effect in improving the chemical properties (except pH) of the acidic soil irrespective of AMF inoculation, although the effect is not major (Table 6). The total N and available P in the soil were significantly improved with increasing levels of bamboo biochar irrespective of AMF inoculation and soil sterilization. The soil without biochar showed the lowest total N, available P, and exchangeable K in either of the two soils, with or without AMF inoculation (Table 6). The organic matter (OM) in the sterilized soil was not affected by BB and AMF, but in unsterilized soil, only 15% BB improved the OM over the other considered treatments. The CEC of the two soils was consistently improved by addition of 15% BB (Table 6).

4. Discussion

The current involvement of Philippines in the cocoa-chocolate global value chain is limited as it primarily acts as an importer of immediate and final products for domestic consumption. The global share of the country in exportation remained low as it produces less than 0.01% in the world market (Department of Trade and Industry, 2017). The primary challenge to Philippine participation in the chain is the production of low volumes of cocoa beans due to farm-level issues, which include soil nutrient deficiency and acidity. Biochar amendment (Lehmann et al., 2011) and arbuscular mycorrhizal fungi (AMF) inoculation (Smith and Read, 2010) are potential alternatives that may improve plant growth and survival in nutrient-deficient acidic soil.

Acidic soil is a threat to various agricultural and farming systems due to the acid deposition on vegetation (Rorison, 1986), which can severely reduce the growth of the crop (Martinsen et al., 2015). These soils are characterized with low pH, low phosphorus (P) availability, and high contents of aluminum (Al) and manganese (Mn), which limit plant growth (Aguilara et al., 2015).

Phosphorus is a poorly available macronutrient essential for plant growth and development and consequently for successful crop yield and ecosystem productivity (Ferrol et al., 2019). Arbuscular mycorrhizal symbioses are one of the most successful and widespread strategies to maximize access of plants to available P (Smith and Read, 2010). AM fungi colonize the root cortex of most plant species and develop an extraradical mycelium which overgrows the nutrient depletion zone of the soil surrounding plant roots. This hyphal network is specialized in the acquisition of low mobility nutrients from soil, particularly P (Ferrol et al., 2019).

The response of plants due to the function of arbuscular mycorrhizal symbioses differ from positive to negative, especially on the interplay between direct P uptake via root epidermis (including root hairs when present) and uptake via the AM fungal pathway (Smith et al., 2011). AMF modify plant response to acidic soil when symbiotically associated with each other through enhanced P acquisition and reduced heavy metal exposure (Aguilera et al., 2015). In this study, the overall P concentration in the leaves was reduced in mycorrhizal plants regardless of biochar amendment. This result may be attributed to the high amount of available P (nearly 27 mg kg^{-1}) in the soil as a result of nutrient sequestration activity of biochar. Similarly, Moreira et al. (2019) observed a reduction of P concentration in mycorrhizal plants when the substrate received a higher level of P fertilization, suggesting that high P contents can usually inhibit the establishment of mycorrhiza according to the nutritional state of the plant and with the mechanisms of symbiotic regulation. The activity of AMF is affected by the varying concentrations of available P in the soil (De Miranda and Harris, 1994), thus affecting the association with plants. In addition, the N and P concentrations were inversely correlated with plant dry weight indicating a nutrient dilution effect (Jarrell and Beverly, 1981). Mycorrhizal plants in this study, gave heavier total plant dry weight than the non-mycorrhizal counterpart but with lower P concentration in unsterilized soil. However, in terms of plant nutrient uptake (the product of nutrient concentration and plant dry weight), the correlation is directly proportional meaning the higher the plant biomass, the higher the nutrient uptake as reported recently by Aggangan et al. (2019b). Aggangan et al. (2019b) have used sterilized acidic soil (same as what was used in the present study) with the same biochar level and AMF. N and P uptakes were higher in mycorrhizal inoculated plants than in non-mycorrhizal plants in no biochar growing medium, suggesting that the AMF enhanced P uptake of cacao plants. This fungal activity is dictated by signaling pathways commonly shared within root symbiosis, which requires activation of hundreds of genes (Chen et al., 2018). Despite the insignificant effect on soil chemical properties of the acidic soil, AMF generally improved the height and stem diameter of the plant. This result corroborates with the study of Wu and Xia (2004) and Chen et al. (2017), suggesting that AMF improved the aerial growth structure of plants. Arbuscular mycorrhizal fungi establish symbiotic relationship with about 80% of terrestrial plants (Berruti et al., 2016; Wang et al., 2017). They are widely reported to exhibit significant positive effects on plant growth by increasing plant survival, enhancing growth and nutrition, improving soil structure and quality, and greater plant re-establishment (Wang, 2017). This study microscopically observed the evidence of AMF species in both roots and rhizosphere, which are prominent in mycorrhizal plant. A wide range of AMF diversity associated to host plant growing in acidic soils, suggest the selective advantage of AMF in allowing the plant to survive in nutrient-deficient natural habitats (Aguilera et al., 2015; Chen et al., 2018).

Table 4
Interaction effects between arbuscular mycorrhizal fungi (AMF) and bamboo biochar (BB) at increasing level (Nil, 7.5 and 15%) on several growth traits and nitrogen and phosphorus concentration of the youngest fully expanded leaves of cacao plants grown in sterilized and unsterilized soil under nursery condition. N = 3.

Source of variation			Leaf dry weight (g plant-1)	Stem dry weight (g plant-1)	Coarse root dry weight (g plant-1)	Fine roots dry weight (g plant-1)	Total plant dry weight (g plant-1)	Nitrogen (N) concentration (mg g-1)	Phosphorus (P) concentration (mg g-1)
	AMF inoculation	BB level (%)							
Sterilized soil	-AMF	Nil	17.84 bc	35.54 d	12.53 bc	1.95 c	67.86 b	19.85 c	1.23 b
		7.5	13.73 d	47.88 cd	14.71 bc	4.14 c	80.47 b	22.35 a	1.44 a
		15	20.18 a	77.15 a	19.32 ab	10.64 a	127.27 a	18.20 d	1.12 c
	Mean -AMF		**17.25 A**	**53.52 B**	**15.52 A**	**5.58 A**	**91.87 B**	**20.13 A**	**1.26 A**
	+AMF	Nil	19.22 ab	61.89 bc	22.67 a	7.87 ab	111.62 a	21.10 b	1.33 ab
		7.5	16.97 c	51.06 c	10.96 c	7.77 ab	86.74 b	19.20 c	1.38 a
		15	20.49 a	74.18 ab	18.60 ab	5.53 bc	118.90 a	19.55 c	1.22 bc
	Mean + AMF		**18.89 A**	**62.38 A**	**17.41 A**	**7.06 A**	**105.75 A**	**19.95 A**	**1.31 A**
Unsterilized soil	-AMF	Nil	14.32 c	33.62 c	9.83 c	3.54 b	61.31 c	20.35 a	1.32 c
		7.5	18.99 b	69.27 ab	19.29 ab	3.68 b	111.21 b	16.85 c	1.41 c
		15	19.64 ab	64.35 b	17.26 ab	3.12 b	104.38 b	16.30 c	1.82 a
	Mean -AMF		**17.65 A**	**55.75 A**	**15.46 A**	**3.45 B**	**92.30 B**	**17.83 B**	**1.52 A**
	+AMF	Nil	18.94 bc	52.69 b	19.06 ab	9.95 a	100.63 b	20.35 a	1.53 b
		7.5	15.55 bc	54.50 b	11.88 bc	6.23 ab	88.14 b	18.15 b	1.16 d
		15	24.22 a	67.95 a	21.09 a	4.22 b	137.47 a	17.85 b	1.38 c
	Mean + AMF		**19.57 A**	**58.38 A**	**17.34 A**	**6.80 A**	**108.75 A**	**18.78 A**	**1.36 B**

Statistical analyses were done separately on sterilized and unsterilized soil.
Abbreviations: without (−) AMF; with (+) AMF.
Means in a column with different small letters (interaction between AMF and BB level) are significantly different from each other using LSD at $p < 0.05$.
Means in a column with different capital letters (main effect of AMF) are significantly different from each other using LSD at $p < 0.05$.

Table 5
Mycorrhizal root colonization and the estimated spore count of arbuscular mycorrhizal fungi (AMF) spores in the rhizosphere of cacao plants grown in sterilized or unsterilized soil as affected by AMF and bamboo biochar (BB) at different levels (Nil, 7.5, and 15%). N = 3.

Soil treatment	Mycorrhizal root infection (%)				Estimated rhizosphere spore count (spores 10 g soil^{-1})			
	Biochar level (%)	-AMF	+AMF	Mean	Biochar level (%)	-AMF	+AMF	Mean
Sterilized soil	Nil	22 d^{ns}	65 ab	43 B*	Nil	92 b**	35 b	63 B***
	7.5	40 cd	63 ab	51 AB	7.5	48 b	566 a	307 A
	15	53 bc	79 a	66 A	15	87 b	652 a	370 A
	Mean***	38 B	69 A		Mean***	76 B	418 A	
Unsterilized soil	Nil	19 d^{ns}	56 ab	37 B*	Nil	83 b*	57 b	70 B**
	7.5	29 cd	82 a	56 AB	7.5	109 ab	188 a	148 A
	15	43 bc	79 a	61 A	15	112 ab	181 a	146 A
	Mean***	30 B	72 A		Mean^{ns}	101 A	141 A	

Statistical analyses were done separately on sterilized and unsterilized soil.
Legend: without (−) AMF; with (+) AMF.
^{ns}, *, ** indicate not significant, significant at $p < 0.05$, $p < 0.01$ $p < 0.001$ confidence level, respectively.
Means with different small letters (interaction between AMF and BB level) are significantly different at $p < 0.05$ using LSD test.
Means with different capital letters (main effect of AMF and BB) are significantly different at $p < 0.05$ using LSD test.

This study observed that AMF had great positive effect on stem diameter, suggesting a potential benefit to cacao growers in the Philippines. Periodic pruning on plant height is employed to regulate the number of branches so that harvesting of fruits could be reached by hand while the harvesters are on the ground. In this case, stem diameter would be a better parameter than plant height in assessing growth response due to treatments. In addition, the AMF inoculants used in the study had been successfully used for bioremediation using *Jatropha curcas* as the host plant and forest tree species such *Pterocarpus indicus, Acacia mangium* and *Eucalyptus urophylla* (Aggangan et al., 2017, 2019a). Moreover, various farmers in the Philippines also used these inoculants to improve crop production, thus developed a promising effect on agricultural and farming systems in the country. Given that these AM fungi are naturally symbiotic with plants, ecological restoration of contaminated areas is possible (Wang, 2017).

Soil sterilization favors propagation of introduced AMF spores while reducing competition due to elimination of native microbial communities (Garrido, 2009). The presence of AMF in the uninoculated controls was most likely brought by the contaminations from water and air. Soil sterilization is effectively used for various agricultural researches to distinguish the association between microbial processes and abiotic reactions in the soil (Berns et al., 2008). It shapes the chemical properties as affected during the sterilization process (Lotrario et al., 1995; Berns et al., 2008; Tan, 2010). Most of the soil chemical properties measured were reduced by soil sterilization (data not shown) but the reduction did not affect the overall growth of the cacao plants due to the help of the treatments.

In this experiment, fine roots, the nutrient absorbing organ of a plant, was better in the unsterilized than in sterilized soil. This implies that the indigenous microbes could have assisted the introduced mycorrhizal

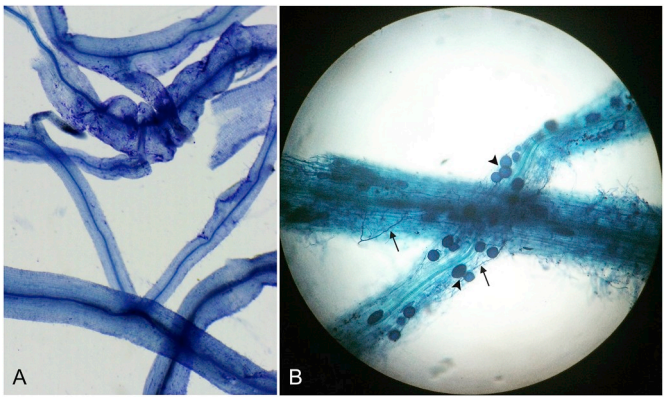

Fig. 5. Representatives of cacao roots without AMF infection (A), and cacao roots infected with arbuscular mycorrhizal fungi (B) showing mycelial hyphae (thick dark blue strands pointed with arrow) and the circular-to oblong-shaped vesicles (the food storage organ of AMF pointed with arrowhead). Cacao fine roots were obtained from the control (A) and from the plant treated with AMF+15% bamboo biochar (B). Total magnification = 100X

fungi. The indigenous microbes could have contributed to the biological processes and physicochemical state of the unsterilized soil, which could have direct impact in the symbiotic association between the plant and fungi (Aggangan and Moon, 2013; Kim et al., 2017). In addition, the overall dry weight of cacao plants in two soils was increased with AMF inoculation, suggesting that AM symbiosis impacted the plant mineral nutrition in any kinds of soil. Studies have shown that the mycorrhizal association between plants and mycorrhizal fungi is accompanied by soil native bacteria, which could be inhibitory or stimulatory to the mycorrhization (Fitter and Garbaye, 1994; Bonfante and Anca, 2009). This study also observed that a high percentage of root infection is not directly or necessarily associated with better plant growth, hence, mycorrhization on plant may vary like those reported by Garrido (2009).

Besides, the use of biochar as a waste management option improves the soil fertility and nutrient cycling, thus enhanced plant growth yield (Carter et al., 2013; Schulz et al., 2013). In this study, bamboo biochar improved most of the growth traits of cacao plants in either sterilized or unsterilized soil possibly due to soil nutrient acquisition, which was similarly noted by Liu et al. (2016). Biochar prevents nutrient leaching and increases water retention due to its high surface area and porous nature, in effect, nutrients are readily available for plant consumption (Batista et al., 2018). Meanwhile, some of the chemical properties of acidic soil were generally improved by biochar amendment, which promoted a better growth of cacao seedlings. Biochar can promote long-term effects on the chemical properties of acidic soil (Chintala et al., 2014) indicating its potential influence on crop growth through soil nutrient acquisition. The changes of the soil chemical properties as affected by biochar amendment could also influence the nutrient cycling by altering the soil microbiota (Xu et al., 2014). These edaphic conditions have been shown in several studies, which revealed the positive effects of biochar amendment on the environment of acidic soil (Lehmann et al., 2011; Carter et al., 2013; Jien and Wang, 2013; Chintala et al., 2014; Xu et al., 2014; Nabavinia et al., 2015; Dume et al., 2016).

Biochar favored an increase in root infection rate (Matsubara et al., 2002) as observed in the study. Higher level of biochar promoted higher root infection rate of AMF resulting into crop growth improvement (Nzanza et al., 2012; Conversa et al., 2015; Liu et al., 2017). Biochar could have protected the introduced AMF from soil predation and competition while being stimulated by native mycorrhiza-helping microbes in the soil, thus improving plant growth (Warnock et al., 2007). These results corroborated with several studies, showing the positive combined effects of biochar and AMF to the growth and yield of the crops (Hammer et al., 2015; Vanek and Lehmann, 2015). On the contrary, there are reports that showed combined applications could be antagonistically and/or did not affect the overall growth of a plant (Nzanza et al., 2012; Liu et al., 2017). Somehow, positive response of plant to AMF could be substantially reduced by biochar amendment (Liu et al., 2017). Nevertheless, this study confirmed the ability of AMF and biochar to improve the overall growth of the crop when grown in acidic soil environment.

Heterogeneity might prevail in biochar application due to differences in raw materials used and pyrolyzing conditions applied (Warnock et al., 2010; Kloss et al., 2012; Zhao et al., 2013; Ramola et al., 2014). These suggest the relative response of plants to the specific type of biochar used in various studies. Plants also respond to AMF inoculation, but the magnitude of response varies mainly due to differences in AMF isolate, soil type and condition (Ndoye et al., 2013; Kim et al., 2017).

5. Conclusion and recommendation

The overall growth of 15-month-old cacao plants was positively altered by AMF and bamboo biochar, the effects of these treatments, however, varied in sterilized and unsterilized soil. The number of mycorrhizal spores in roots and soil increased upon treatment. Soil chemical properties were generally affected by biochar amendment. The positive results of the study may suggest a potential benefit in enhancing soil fertility and crop growth and survival particularly in the Philippines. Farmers and various industries such as those related to cacao can benefit from these results, which can contribute to the agriculture and economic development in the country. Field trials are on-going on seven sites in the CALABARZON Region (Luzon island, Philippines) to verify the above results as the conditions inside a screenhouse is far different from

Table 6
Changes on acidic soil chemical properties as affected by the interaction between AMF inoculation and biochar amendment at different level (Nil, 7.5. and 15%) in sterilized and unsterilized acidic soil after 15 months under nursery conditions. N = 3.

Source of variation		pH		N (g kg^{-1})		P (mg kg^{-1})		K (me 100 g soil^{-1})		OM (g C kg soil^{-1})		CEC (me 100 g soil^{-1})	
AMF treatment	Biochar level (%)	S	US	S	US	S	US	S	US	S	US	S	US
-AMF	Nil	4.7 a	4.8 a	0.4 d	0.6 d	2.60 e	3.90 e	0.24 d	0.22 d	12.3 b	12.8 bc	11.08 bc	11.38 c
	7.5	4.8 a	5.1 a	1.1 c	1.1 c	6.80 c	12.00 d	2.06 c	1.74 b	19.7 ab	25.7 c	10.48 c	11.79 c
	15	5.1 a	4.9 a	1.4 b	2.2 a	11.25 a	16.75 c	3.80 a	1.34 c	24.4 ab	78.7 a	12.42 a	14.01 a
	Mean -AMF	**4.87**	**4.93**	**1.0**	**1.3**	**6.88**	**10.88**	**2.03**	**1.10**	**18.8**	**39.1**	**11.33**	**12.39**
+AMF	Nil	4.7 a	4.7 a	0.6 d	0.5 d	3.00 e	3.65 e	0.15 d	0.25 d	11.0 b	9.5 c	11.52 b	13.18 b
	7.5	4.9 a	4.9 a	1.1 c	1.0 c	5.25 d	22.50 b	2.22 c	1.27 c	35.0 a	22.2 bc	11.68 b	11.68 c
	15	5.0 a	4.8 a	1.6 a	1.8 b	9.10 b	26.85 a	3.14 b	2.85 a	25.5 ab	20.3 bc	13.11 a	13.83 a
	Mean + AMF	**4.87**	**4.87**	**1.1**	**1.1**	**5.78**	**17.67**	**1.84**	**1.46**	**23.8**	**17.3**	**12.1**	**12.90**

Statistical analyses were done separately on sterilized and unsterilized soil.
Legend: +AMF = with arbuscular mycorrhizal fungi; -AMF = without arbuscular mycorrhizal fungi; S = sterilized soil; US = unsterilized soil.
Means in a column with different letters are significantly different from each other using LSD at $p < 0.05$.

that in the field. Recommendation to farmers for adoption of the technology will be based on the results of field trials coupled with demonstration plots in farmers' field.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101347.

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