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Characterization of Plant Growth Promoting Diazotrophic Bacteria Isolated from Cacao (*Theobroma cacao* L.) Rhizosphere Treated with Bamboo Biochar and Arbuscular Mycorrhizal Fungi

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Diazotrophic bacteria (syn. nitrogen-fixing bacteria, NFB) are plant growth-promoting rhizobacteria known to facilitate nitrogen availability for plant nutrition. Biochar may serve as a refuge for colonizing microbes influencing the mycorrhizal richness and activities in the rhizosphere, thereby affecting the mycorrhization helping bacteria. This study isolated, characterized, and identified diazotrophic bacteria from the cacao (*Theobroma cacao* L.) rhizosphere treated with arbuscular mycorrhizal fungi (AMF) and bamboo biochar (BB). Initial screening of the cacao rhizosphere yielded 1.28x10^6 CFU of diazotrophic bacteria g^{-1} soil grown in nitrogen-free malate medium. A total of 12 isolates were further assayed for acetylene reduction and phosphate solubilization activities. All putative diazotrophic bacterial isolates successfully reduced acetylene to ethylene gas, ranging from 16.35 ± 1.68 to 60.71 ± 2.57 ppm. Of the 12 putative diazotrophic bacterial isolates, 11 have shown phosphate solubilization activity on Pikovskaya's medium with the solubilization index (SI) ranging from 1.12 ± 0.08 to 3.74 ± 0.16 mm. Analysis of the 16S rRNA gene sequences revealed that these diazotrophic bacterial isolates belonged to the genera *Bacillus*, *Burkholderia*, *Rhizobium*, and *Ralstonia*. These results provide evidence of candidate plant growth-promoting rhizobacteria from cacao that are potential source of biofertilizers to improve the growth and yield of cacao crops, especially in the Philippines. As agricultural options, biochar and AMF pose beneficial influence on plant growth-promoting bacteria, which primarily provide nutrients for several crops.

Keywords: arbuscular mycorrhizal fungi, bamboo biochar, cacao rhizosphere, diazotrophic bacteria, plant growth-promoting rhizobacteria

INTRODUCTION

Nitrogen (N) is the most critical limiting key element for plant nutrition (White and Brown 2010). It is an important precursor for various metabolic cellular processes of plants such as photosynthesis, energy transfer, signal transduction, and macromolecular synthesis (Halbleib and Ludden 2000; Sharma *et al.* 2013). However, atmospheric N_2 has a very stable strong triple bond where its diatomic form makes it inaccessible for plant nutrition; therefore, it must be fixed by a small number of specialized N_2-fixing microbes such as diazotrophs (Galloway *et al.* 2003; Eskin *et al.* 2014). Diazotrophic bacteria, also known as nitrogen-fixing

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bacteria (NFB), are important microbes that fix atmospheric N_2 gas into a usable form like ammonia (NH_3) and readily absorbed by plants to participate in various physiological processes (Raymond *et al.* 2004; Wagner 2011). Dinitrogen (N_2) fixation is driven by a highly conserved nitrogenase enzyme complex wherein activation is controlled in the absence of oxygen both in free-living and symbiotic diazotrophs (Raymond *et al.* 2004; Santi *et al.* 2013). These microorganisms offer potential nutrient gains to many plants for growth and development. Diazotrophic bacteria may perform symbiotic or non-symbiotic relationship with plants depending on their individual functions in the environment (Smercina *et al.* 2019). Non-symbiotic diazotrophic bacteria are free-living bacteria that fix free nitrogen in the soil, while symbiotic ones are capable of building symbiosis to establish a close long-term relationship with the plants for their nutrition – an example of which is nodule formation where nitrogen fixation occurs (Franche *et al.* 2009; Smercina *et al.* 2019). Diazotrophic bacteria are plant growth-promoting rhizobacteria that perform other attributes such as phosphate solubilization and indolic compounds production, indicating potential benefits in crop production (Venieraki *et al.* 2011). Besides, a previous study assessed the nitrogenase activity of the free-living diazotrophic bacteria isolated from rhizosphere of different agricultural crops – namely, sesame, maize, wheat, soybean, lettuce, pepper, and rice (Park *et al.* 2005) – but a study on the cacao rhizosphere is limited.

Cacao (*Theobroma cacao* L.) crop is a typical monoecious and cross-pollinated plant that produces an indehiscent berry-type fruit (Lachenaud *et al.* 2007). The cacao crop is now on high demand worldwide yet it faces some big challenges especially in cultivation, and farmers often need support to make their farm more productive and profitable. In the Philippines, the production of cacao reached up to 6,020 metric tons at 13,910 ha based on the 2015 data of Philippine Statistics Authority (BPI-DA 2017). Besides, the country's exports of cocoa products remain low with a global market share of less than 0.01% (DTI 2017). To improve the production of cacao seedlings grown in acidic soil environment, soil conditioners and biofertilizers are suggested – including the application of biochar and AMF, respectively. Biochar is a solid product of biomass pyrolysis that has diverse applications, including as soil amendment to improve soil fertility and sequestrate carbon (Weber and Quicker 2018). On the other hand, AMF are natural root symbionts that provide essential plant inorganic nutrients to the host plants, thereby improving growth and yield under unstressed and stressed environments. Inoculation of AMF provides tolerance to host plants against various stressful conditions like heat, salinity, drought, metals, and extreme temperatures (Begum *et al.* 2019). These agricultural means not only affect the soil physico-chemical properties and growth of crops but also influence the biological properties of the rhizosphere (Filion *et al.* 1999; Kolton *et al.* 2011). Diazotrophic bacteria are reportedly being stimulated by these agricultural means; thus, bacterial isolation and characterization is important to assess their potentials as biofertilizer in improving the growth of cacao seedlings.

This study aimed to characterize diazotrophic bacterial isolates from cacao rhizosphere treated with biochar and AMF by assessing their acetylene reduction and phosphate solubilization activities. The 16S rRNA gene sequences of the isolates were amplified and sequenced to confirm their identity.

MATERIALS AND METHODS

Experimental Setup and Cultivation of Seedlings

The two-week old pre-germinated cacao (UF18) seedlings were transferred (one seedling per bag) into individual expanded (4" x 10" x 2") black polyethylene bags filled with soil: biochar (1700 g: 300 g) mixture (corresponding to 15% biochar). Seedlings were inoculated with 5 g of soil mycorrhizal inoculant consisting of 12 species belonging to genera *Glomus*, *Gigaspora*, *Entrophospora*, and *Acaulospora* placed directly beneath the roots. The preparation of BB and AMF inoculants was previously described by Aggangan and colleagues (2019b).

Cacao seedlings were placed on steel benches (1 m width and 5 m length) half-meter off the ground inside the nursery (average temperature of 27.5 °C). Watering was done by weight (field capacity) and polybags were rearranged once a week to minimize any non-homogenous penetration of sunlight. One gram (1 g) of NPK fertilizer was applied to each plant once per month during the first two months after planting. Seedlings were grown for 15 mo under nursery conditions at the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños.

Sample Collection and Isolation of Diazotrophic Bacteria

After 15 mo, a total of 100 g composite samples of rhizospheric soil (pH 4.8) from the three pots of cacao rhizosphere treated with 15% BB and AMF were collected. Isolation of diazotrophic bacteria was carried out using a nitrogen-free malate medium (Estrada-de los Santos *et al.* 2001), following serial dilution technique and spread plating method. The population count of culturable diazotrophic bacteria yielded about 1.28×10^6 CFU g $soil^{-1}$ (fresh weight), as initially screened by Aggangan and colleagues (2019a). The diazotrophic bacteria with unique

culturable characteristics (such as color and shape) were selected, streaked, and purified on tryptic soy agar broth (TSB) medium. Bacterial isolates were then incubated at 37 °C for 24 h during isolation and then properly stored at –80 °C in a glycerol stock for further use.

Assessment of the Plant Growth Promoting Attributes of the Diazotrophic Bacterial Isolates

Twelve (12) diazotrophic bacteria were selected from the preliminary screening assay to assess their metabolic activities such as acetylene reduction and phosphate solubilization.

Acetylene reduction assay. The nitrogenase activity of the isolates was tested using acetylene reduction assay (ARA), as previously described by Venieraki *et al.* (2011). Each bacterial isolate was stabbed into an 8-mL semisolid nitrogen-free malate medium in a 15-mL tube and incubated for 24–30 h at 30 °C. After incubation, each tube was then sealed aseptically with sterile rubber septum and 2 mL of air were replaced with the same volume of acetylene gas using a sterile 1-mL disposable syringe. The isolates were further incubated for 20–24 h at 30 °C. The amount of ethylene produced by acetylene reduction process was measured using a Shimadzu 14B gas chromatograph fitted with a flame ionization detector and a Porapak N 80-100 mesh stainless-steel column (1.1 mm x 2.1 m). Ethylene gas standard was prepared using 1 ppm, 10 ppm, 100 ppm, and 1000 ppm of ethylene gas. For the assay, *Azospirillum* sp. was used as the control, as it has been reported to benefit plants through biological nitrogen fixation (Fukami *et al.* 2018).

Phosphate solubilization assay. The phosphate solubilization activity of the diazotrophic bacterial isolates was determined using spot plate method on Pikovskaya's medium, as described by Venieraki and colleagues (2011). Bacterial isolates were grown in 1.5 mL TSB in a 2-mL tube at 37 °C with shaking for 48 h. After incubation, 4 µL of 24-h bacterial broth suspension was then spotted on Pikovskaya's agar plates in triplicates, inverted, and incubated up to 9 d at 30 °C. The clear halo zone around the colonies was monitored and the SI was measured by dividing the diameter of colony + halozone over diameter of the colony, as described by Kumar and Narula (1999). For this assay, *Pseudomonas aeruginosa* was used as the control on account of their phosphate solubilization ability (Linu *et al.* 2019).

Statistical Analysis

The data from the ethylene production and phosphate SI were analyzed for statistical significance through analysis of variance with three replicates of bacterial isolates. Mean values were then compared using Tukey's Test at $P \leq 0.05$ through MSTATC package.

Molecular Identification of Diazotrophic Bacterial Isolates by Analysis of 16S rRNA Gene Sequence

Extraction of genomic DNA. DNA was extracted and purified from diazotrophic bacterial isolates using CTAB (cetyltrimethylammonium bromide) method adapted from the DOE Joint Genome Institute protocol (version 3 by William S. Feil, Helena Feil, and A. Copeland). A 24-h broth culture was prepared, and cells were collected by centrifugation at 12,000 rpm for 45 s. The supernatant was discarded, and the pellet was resuspended in 200 µL of 1x TE buffer using a vortex. A volume of 25 µL of 10 % SDS and 5 µL of proteinase K (20 mg mL^{-1}) was added to the resuspended cells consecutively and was incubated at 37 °C for 1 h. After incubation, 45 µL of 5 M NaCl and 40 µL of CTAB/NaCl solution (10 %: 0.7 M) were added to the micro centrifuge tubes (2 mL), mixed by inverting the tubes, and allowed to stand at 37 °C for 20 min. An equal volume of chloroform: isoamyl alcohol (24:1) was then added into the mixture. The tubes were allowed to stand for at least 30 min after mixing. Aqueous phase was separated from the organic phase through centrifugation at 15,000 rpm for 10 min. The aqueous phase was placed into the fresh 2 mL tubes and then treated with RNAse plus an equal volume of 100% ethanol. DNA was precipitated by centrifugation at 15,000 xg for 10 min, the supernatant was discarded, and the pellet was resuspended again into an equal volume of 70 % ethanol. The mixture was further centrifuged at 14,000 rpm for 5 min and the supernatant was removed and discarded. The pellet was air-dried for 5 min and immediately resuspended into 100 µL of 1x TE buffer.

Isolated genomic DNA was verified using MUPID-2 Plus gel electrophoresis (Gel Company, San Francisco, USA). Briefly, 0.8 % agarose powder was dissolved in 50 mL of 0.5x TAE buffer. Cooling agarose gel was stained by adding 1.25 µL of GelRed® dye and poured into the casting tray with comb. Five microliters (5 µL) of genomic DNA was mixed in 3 µL of 2x loading dye and immediately placed into the well and run under 100 V for 30 min. DNA samples on the gel were viewed using Gel Doc scanner. DNA concentration and purity were assessed using Epoch™ microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

PCR amplification and sequencing of 16S rRNA gene. The 16S rRNA gene region was PCR (polymerase chain reaction) amplified in all 12 characterized diazotrophic bacterial isolates using the primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3'; Lane 1991) and 1492r (5'-GGTTACCTTGTTACGACTT-3'; Lane 1991). PCR was performed in a 50-uL reaction containing the following: 1x PCR buffer (Invitrogen); 2 mM $MgCl_2$; 0.2 µM of 27f and 0.2 µM of 1492r primers; 0.2 mM dNTP

(Invitrogen); 1.5 U μL^{-1} DNA polymerase (Brazilian *Taq* polymerase, Invitrogen); and 100 ng of DNA template. A final volume of 50 µL was adjusted with molecular grade water. PCR reactions were run using thermocycler (BIORAD®) with the following conditions: initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s, and 72 °C for 1 min, with a final extension step of 72 °C for 10 min. The PCR products were verified through gel electrophoresis using 1.2 % agarose gel (stained with GelRed® dye) under 100 V for 35 min.

The PCR products were sent to Apical Scientific Sdn Bhd (Selangor, Malaysia) for Sanger sequencing using universal bacterial primer 27f. The electropherogram of the sequences was inspected and verified using Applied Biosystems' Sequence Scanner Software v2.0 (Thermo Fisher Scientific, USA).

16S rRNA sequence analysis of individual diazotrophic bacterial isolate. Test strain sequences obtained from Apical Scientific (Selangor, Malaysia) were trimmed to remove the unwanted characters from the start and the end of the sequences using Molecular Evolutionary Genetics Analysis (MEGA) version 10.0.4: TraceEditor. Trimmed sequences were individually compared with the non-redundant collection of sequences in the Genbank database using Basic Local Alignment Sequence Tool (BLASTn) to determine the identity and sequence homologies of the unknown isolates (Altschul *et al.* 1990). BLASTn hits were recorded and the highest (≥ 97 %) similarity was verified. Also, the 16S rRNA gene sequences of test strains were compared against those sequences in Ribosomal Database Project (RDP) by using the RDP Classifier Check Program to give sequence matches of candidate species identity of the unknown up to the genus level (Cole *et al.* 2014).

The partial sequences of 16S rRNA available in the NCBI database with ≥ 97% similarity of each test strain sequence were downloaded and aligned with the unknown sequence together with the outgroup. Multiple sequence alignment for the homologous sequences was carried out using the algorithm described in MUSCLE of M7: Alignment Explorer. Subsequently, evolutionary distance matrix was generated from the nucleotide sequence in the dataset. The phylogenetic relationship between sequences were constructed through neighbor-joining method with 1000 replicates bootstrap values to estimate the confidence level of tree topologies. Phylogenetic and molecular evolutionary analyses were carried out using MEGA 7 (Kumar *et al.* 2016). The annotated information for the sequence in the database, to which 16S rRNA aligns is used for the bacterial identification.

RESULTS AND DISCUSSION

Plant Growth Promoting Attributes of Diazotrophic Bacteria

Acetylene reduction assay. Based on the results, all diazotrophic bacterial isolates reduced the acetylene to ethylene gas and the produced ethylene gas ranged from 16.35–60.71 ppm. The lowest ethylene produced was detected in isolate 3, while the highest ethylene produced was observed for isolate 6. Approximately 75% of the diazotrophic bacterials isolates exhibited higher activity of acetylene reduction compared to *Azospirillum sp.* as the control (22.73 ppm) (Table 1), indicating a potential role to be developed as biofertilizer. Atmospheric N_2 fixation is driven by a highly conserved nitrogenase enzyme complex that is commonly possessed by NFB (Raymond *et al.* 2004). Nitrogenase is also capable of reducing acetylene gas into ethylene gas, which can be measured using gas chromatography *in vitro*. Acetylene reduction is the common way of determining the nitrogenase activity of an organism in a highly sensitive and inexpensive way (David *et al.* 1980).

Phosphate solubilization index. The study demonstrated that 11 out of the 12 diazotrophic strains also exhibited phosphate solubilization on Pikovskaya's agar medium based on the clear halo zone observed around the colonies (Figure 1). The phosphate SI observed varied from 1.12 ± 0.08 to 3.74 ± 0.16 mm. Highest SI (3.74 mm) was observed in isolate 1, which is 56 % higher than the control, 2.40 mm. Diazotrophic bacterial isolate 1 was also found to fix atmospheric nitrogen, thus considered as very promising and could potentially be used for biotechnology. Lowest SI value was observed in isolate 3, whereas no phosphate solubilization activity was observed in isolate 10 (Table 1). Phosphate solubilizing microorganisms solubilize insoluble P through production of organic acids and chelating substances (Kumar and Narula 1999). This activity plays a vital role in natural biogeochemical processes to provide plants with nutrients, thus improving crop production.

As mycorrhizal helping bacteria (MHB), diazotrophic bacteria and phosphate solubilizing bacteria are primarily involved in nitrogen and phosphorus cycling, respectively. These bacteria usually interact with mycorrhizal fungi to develop the so-called "mycorrhizosphere" that is fundamental in sustainable crop production (Azcón-Aguilar and Barea 2015). The MHB stimulate mycelial growth and mycorrhiza formation, thus acting as plant growth promoters, which improve fungal colonization of fine roots due to plant cell wall modifying bacterial substances (*e.g.* auxin and IAA) (Labbé *et al.* 2014).

Table 1. Molecular identity of diazotrophic bacterial isolates based on their 16S rRNA gene using BLAST and MEGA7 and their corresponding ethylene production and phosphate SI.

Isolate	Identity (≥ 97%)	BLAST highest match (%)	Ethylene produced (ppm)	Phosphate SI (mm)
1	*Burkholderia* sp.	97	21.32 ± 0.41 [g]	3.74 ± 0.16 [a]
2	*Bacillus* sp.	98	29.53 ± 2.39 [d]	1.36 ± 0.05 [d]
3	*Bacillus* sp.	99	16.35 ± 1.68 [h]	1.12 ± 0.08 [e]
4	*Rhizobium* sp.	98	42.78 ± 3.18 [c]	1.17 ± 0.02 [e]
5	*Bacillus subtilis*	100	44.80 ± 1.73 [c]	1.17 ± 0.09 [e]
6	*Bacillus xiamenensis*	98	60.71 ± 2.57 [a]	1.23 ± 0.03 [e]
7	*Bacillus subtilis*	97	25.21 ± 0.94 [ef]	1.75 ± 0.10 [c]
8	*Ralstonia mannitolilytica*	97	45.78 ± 0.74 [c]	1.41 ± 0.11 [d]
9	*Rhizobium* sp.	98	23.22 ± 1.12 [fg]	1.15 ± 0.03 [e]
10	*Bacillus cereus*	99	20.94 ± 0.70 [g]	–
11	*Rhizobium* sp.	98	52.00 ± 1.59 [b]	1.18 ± 0.08 [e]
12	*Rhizobium miluonense*	99	26.89 ± 2.21 [de]	1.20 ± 0.03 [e]
Control[1]			22.73 ± 1.88 [g]	ND
Control[2]			ND	2.4 ± 0.07 [b]

[a]Means with different letters (a–g) are significantly different with each other based on T-test at $P \leq 0.05$.
[b]Legend: not determined (ND); no activity (–).
[c][1]*Azospirillum* sp.; [2]*Pseudomonas aeruginosa*

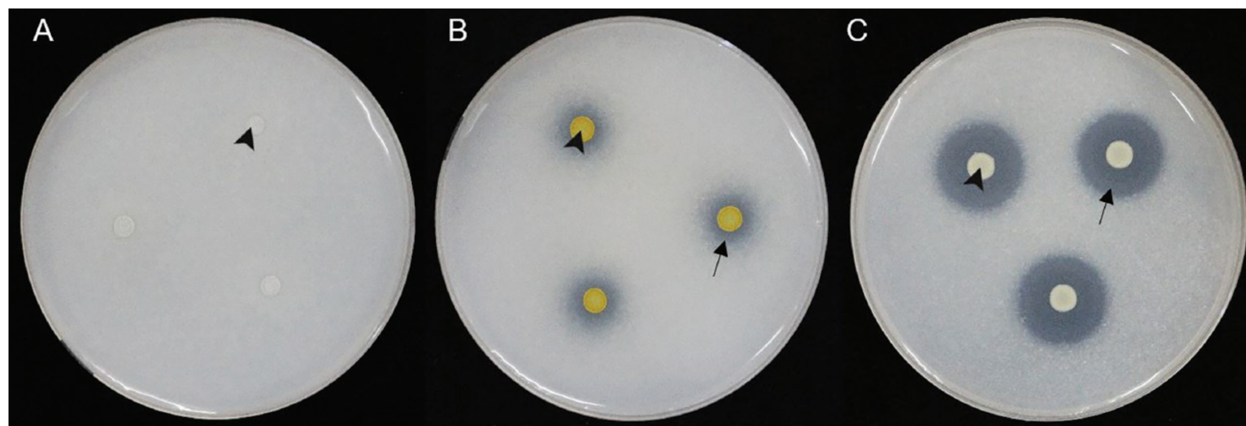

Figure 1. Diazotrophic bacterial isolates screened for phosphate solubilization activity on Pikovskaya's medium with an incubation period of 9 d at 30 °C. Representatives include (A) without activity or no clearing from isolate 10, (B) positive control *Pseudomonas aeruginosa*, and (C) with activity or clearing by isolate 1. Arrow heads pointed the bacterial colonies, whereas arrows pointed the clearing around colonies.

These plant growth-promoting attributes of microorganisms are promising in terms of improving the overall growth of various plants. The diazotrophic bacterial isolates obtained from the cacao rhizosphere with AMF + 15 % BB might have played an important role in improving the overall growth of the host plant in the present study. The identity of these isolates will provide information on which culturable species of NFB in the cacao rhizosphere were influenced by the combined treatment.

Diazotrophic Bacterial Identification

Results revealed that the 12 diazotrophic bacteria isolated from the rhizosphere of cacao seedlings treated with AMF + 15 % BB belonged to the genera *Bacillus*, *Burkholderia*, *Rhizobium*, and *Ralstonia* based on the 16S rRNA gene sequence analyses with at least 97% BLAST highest match (Table 1). These diazotrophic bacterial isolates successfully reduced the acetylene gas into ethylene gas, and most of them were able to solubilize inorganic phosphate (Table 1). Isolate 1 was identified

as *Burkholderia* sp. The genus *Burkholderia* has wide geographic distribution and reported to have substantial capability to fix atmospheric N_2 (Estrada-de los Santos *et al.* 2001). In the present study, the *Burkholderia* species performed nitrogen fixation and phosphate solubilization. *Burkholderia* sp. was found to have the highest phosphate SI compared to other diazotrophic bacterial isolates including the control (*Pseudomonas aeruginosa*) (Table 1). In a related study, *Burkholderia* species was found to promote plant growth and suggested as good candidate to be applied as biofertilizer and biocontrol agent under wide range of environmental conditions (Zhao *et al.* 2014).

Six diazotrophic bacterial isolates were found to belong to the genus *Bacillus*. Of these, two *Bacillus* species including isolates 5 and 7 were both identified as *Bacillus subtilis*, two were identified individually as *Bacillus xiamenensis* and *Bacillus cereus*, while the rest were identified as *Bacillus* sp. The genus *Bacillus* has showed wide range of biological control with several important attributes such as crop growth promotion, nitrogen fixation, and plant nutrient uptake stimulation (Shafi *et al.* 2017). A recent study observed that some *Bacillus* species have showed plant-growth promoting attributes – including nitrogen fixation, phosphate solubilization, and auxin production (Batool *et al.* 2016). However, among the six *Bacillus* strains isolated from the rhizosphere of cacao, only *B. cereus* did not perform phosphate solubilization (Table 1).

On the other hand, four diazotrophic bacterial isolates (4, 9, 11, and 12) were also identified to belong to the commonly known genus of nitrogen fixers – the genus *Rhizobium.* These *Rhizobium* isolates elicit nodule formation where nitrogen fixation occurs, and its species or groups are classified according to their legume host range (Child 1975). The *Rhizobium* species also regulates the production of Nod factors under salt stress in a flavonoid-independent manner (del Cerro *et al.* 2017).

Lastly, isolate 8 was identified as *Ralstonia mannitolilytica*, which was recently found to have the wide range activities as plant growth-promoting bacteria such as nitrogen fixation and phosphate solubilization (Paul and Datta 2016), similarly noted by the present study (Table 1). This biological agent is promising for biotechnology use especially in heavy metal contaminated areas, such as those with increasing concentration of cadmium, nickel, and zinc (Paul and Datta 2016).

Overall, this study demonstrated that the characterized and identified diazotrophic bacteria, obtained from cacao rhizosphere treated with biochar and AMF, showed good potentials to be used as biofertilizers. Free-living nitrogen fixation in the rhizosphere is ubiquitous and a significant source of nitrogen in some terrestrial systems, implying an interest in crop production as an alternative to chemical fertilizers (Smercina *et al.* 2019). As an agricultural amendment, biochar was reported to increase the *nifH* gene abundance and altered the community structure of soil diazotrophs (Liu *et al.* 2019). On the other hand, AMF establish mycorrhizosphere when their extraradical mycelium associates with diazotrophic microorganisms (Trabelsi and Mhamdi 2013). In the rhizosphere, MHB interact with each other to promote mycorrhization of plants, which can be induced by stimulating mycelial growth, by increasing root-fungus recognition and colonization and by reducing the effects of hostile environmental factors (Jambon *et al.* 2018). Biochar may serve as a source of organic compounds and as a refuge for any biochar colonizing microorganisms, protecting them from soil predators (Warnock *et al.* 2007).

CONCLUSION

All putative diazotrophic bacterial isolates obtained from acidic cacao rhizosphere treated with biochar and AMF reduced the acetylene gas to ethylene gas, confirming its nitrogenase activity. Of these, only one isolate did not solubilize the phosphate in the medium. Molecular identification confirmed that these isolates belong to the genera *Bacillus*, *Burkholderia*, *Rhizobium*, and *Ralstonia.* These genera include species that can fix and solubilize important compounds such as N_2 and phosphate, respectively. These diazotrophic isolates can be developed as potential biofertilizers to improve cacao growth and yield here in the Philippines.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interests on this publication.

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