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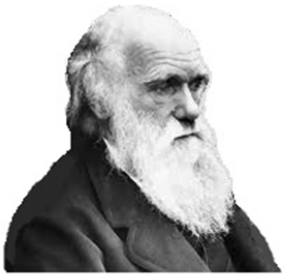

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Isolation and Characterization of Biofertilizer Microbes within Rice Rhizosphere of Soil Samples Collected from Different Agroecological Zones of Ebonyi State, Nigeria, for Enhanced Plant Growth

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ABSTRACT

Rhizosphere microbial community plays a critical role in plant growth and development, serving as biofertilizer that solubilizes minerals, enhances nutrient uptake, and produces plant growth-promoting substances. Therefore, this study was caried out to isolate and characterize the microbial community structure in flooded Paddy across Ebonyi State Agroecological Zones, for Biofertizer microbes and effects on Rice plant growth. The four rice accessions (Oryza sativa) used in this study were sourced from Biotechnology Research and Development Centre, Ebonyi State University; and planted in Pots with control checks (unplanted soil), in a Greenhouse. The physicochemical parameters of the soil were determined using AOAC standard methods. The microbial community in the rhizosphere of paddy microcosms was characterized using (Colony counting machine, expressed in colony forming unit per mililitre (cfu/ml)), 16SrRNA gene amplification and three Random Amplified Polymorphic DNA (RAPD) primers namely, OPB05, OPT05, and OPB03 and resolved independently on agarose gel. Rice plant growth were measured in heights across four growth stages and across the three Agroecological Zones. The physicochemical properties showed that the soil was slightly acidic, had different fractions

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of sand, Organic Carbon, Organic matter and essential nutrients such as; Ca, P, and Mg. The microbial population differed significantly between planted and unplanted soils; while the microbial load decreased gradually as the plants' growth stages increased. Isolates biochemical analysis revealed mostly the presence of bacteria (Bacillus and Brevibacillus species), while confirmatory molecular identification of the bacterial isolates, showed that Aeromonas hydrophilia strain A210 16S, Pseudomonads plecoglossicida strain RJ39 16S, Aeromonas caviae 16S, Eschericha coli strain 26561, Pseudomonas otitidis strain JK79 16S, Enterobacter cloacae strain ES-2 16S, Serratia marcescens strain AL105_R2A02 16S and Aeromonas diversa strain 2478-85 16S were present in the rhizosphere of different Oryza species. Phylogenetic relatedness among the isolates showed isolates B1, B3 and B10 to be closely related. It showed that 90% of the isolates were of the gamma proteobacteria origin, while 10% belong to the Firmicutes. On 90 Day After Planting (DAP), Faro 59 performed the highest across the zones, while Faro 52 was the least performed across the zones. The study has proven the presence of biofertilizer microbes in rice rhizosphere and that; Despite a higher absolute microbial abundance, there was no major shift in the relative abundance of microbial groups in the planted paddy, suggesting how highly adapted and relatively stable, and beneficial microbial community is to rice plants across Ebonyi State soil, and the possibilities of using them as biofertilizer source by farmers and breeders as a sustainable alternative to chemical fertilizer in promoting plants growth and yield to mitigate environmental impacts.

***Keywords*:** Rhizosphere, Paddy, Biofertizer, Agroecological zones, Oryza

1. INTRODUCTION

Microorganisms in the soil or on the surface of the root exerts beneficial effects on plant growth, microbes participates in environmental practices such as the biogeochemical cycling of nutrients and matter, plant health management, maintenance of soil quality, removal of toxins and others, hence, their diversity is important (Barea et al., 2005). Rice plant presents a natural habitat to assorted microorganisms, those that colonize the aerial parts (phyllosphere), the root surface (rhizoplane) and the zone around the root (rhizosphere) (Compant et al., 2013). The root area serves as a source of excretion of different organic compounds into the sink, which is the rhizosphere; hence, the rhizosphere contains solution-phase, volatile, and gas phase compounds. From the microbial perspective, the rhizosphere is a rich zone of nutrients. The totality and active zone of the rhizosphere is not defined, but goes up to few millimeters and relies upon the type of soil and roots. The study of microorganisms in the rhizosphere and their interaction with plant roots is a complex task compared with the study of microbes and microbial interactions in bulk soil. Guo et al. (2024) examine the variations in the surrounding bulk soil and the rice rhizosphere microbiome in 50 locations in eastern China. The dynamics sheds light on sustainable farming methods and emphasizes the role that the rhizosphere bacteria play in low-oxygen rice production. The study highlights the distinct ecological activities of the rice rhizosphere microbiome in improving crop productivity and soil functioning.

Chen et al. (2022) uses slow-release fertilizers to examine the variety of microorganisms in the rice rhizosphere under different fertilization regimes. Three ways of fertilization are compared in this study: mechanical deep implantation at a higher rate (SF), somewhat lower rate (DSF), and standard surface broadcasting (CK). According to the research, deep-planting

slow-release fertilizers is a useful tactic for boosting rice productivity and microbiological diversity while encouraging environmentally friendly farming methods.

The study by Wu et al. (2021) looks into how different kinds of controlled- and slow-release fertilizers affect rice yield. The study comes to the conclusion that using controlled- and slow-release fertilizers in agricultural systems can promote environmental sustainability while increasing rice yields. The properties and processes influencing the biodegradation of controlled-release fertilizers (CRF) and slow-release fertilizers (SRF) made using agro-biopolymers are investigated by Firmanda et al. (2023). The authors emphasize that the addition of biopolymers does not always promote biodegradability, concluding that the interaction of these properties; material composition, crystallinity, additives and surface charge, composite thickness and fertilizer concentration, particle size and hydrophilic-hydrophobic interactions, porosity as well as cross-linking can either enhance or inhibit biodegradation. The rhizosphere is of central attention, not just because of plant nutrition, health and quality but also for microbe-driven carbon sequestration, ecosystem functioning and nutrient cycling in terrestrial ecosystem (Singh *et al.*, 2004).

Rice differs from most crops in that it is regularly cultivated in flooded soil, giving rise to an oxic and anoxic zones within the rice rhizosphere that select for a given physiological groups of microorganisms with aerobic (oxygen consuming), anaerobic, or facultative metabolism (Brune et al., 2000). The major shifts in Northeast China's paddy rice agriculture from 2000 to 2017 are examined by Xin et al. (2020).

The study links agricultural expansion to wider ecological and food security consequences by highlighting the quick rise of paddy rice production and regions in Northeast China. Numerous studies have been carried out on the microbial communities within the rice field ecosystem, for example, the microbes within the rice root interior, the rhizoplane and the rhizosphere have been characterized (Edwards et al., 2015). More studies have investigated the rice phyllosphere microbial community by 16S rRNApyrotag sequencing (Ren et al., 2014) just as endophytic and rhizospheric communities with metagenomic and metaproteomic approaches (Knief *et al.*, 2011).

Biofertilizers have emerged as a sustainable alternative to chemical fertilizers, promoting plant growth and yield while mitigating environmental impacts. Biofertizer of selected efficiency and living microbial culture, when applied to plant surfaces, seed or soil, can colonize the rhizosphere or the interior part of the host plant and afterward promote plant growth by increasing the availability, supply, or up-take of basic nutrients by the host. Additionally, in contrast to chemical fertilizers, biofertilizers are more available/or accessible to marginal and small-scale farmers. The main groups of microbes utilized in the preparation of microbial biofertilizer are bacteria, fungi, and cyanobacteria, majority of which have symbiotic relationship with plants.

The important types of microbial fertilizers based on their nature and function, are those which supply nitrogen and phosphorus. Experimental results have revealed the bacteria and archaea that feed on plant-derived carbon in the rhizosphere and help in nitrogen fixation and phosphate solubilizing (Hernández et al., 2015). Notwithstanding, little or none of these investigations have been carried out in Ebonyi State, Nigeria particularly utilizing molecular approach in characterizing the rhizosphere soil of rice plants. Culturally-based techniques have been attempted by a few researchers studying rice microbial ecology. Hence, this study aimed to screen and characterize biofertilizer microbes for their potential to enhance plant growth and yield.

2. MATERIALS AND METHODS

2. 1. Study Area

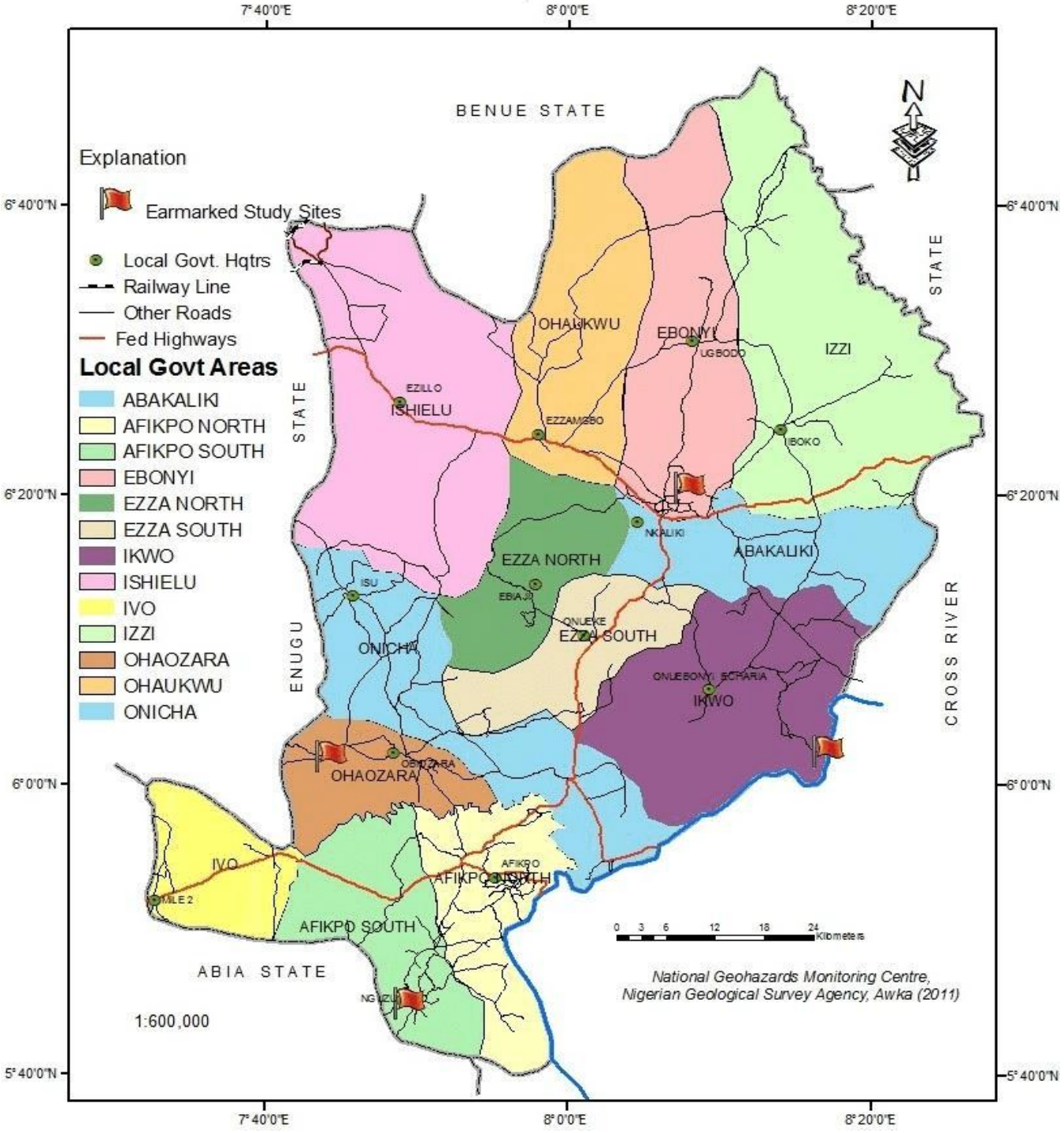

Figure 1. Map of Ebonyi State showing the Agroecological zones of Ebonyi North, Ebonyi Central and Ebonyi South.

The sampled areas include three districts (viz-a-viz; Ebonyi North (Abakaliki with geographical coordinate, 6.3231° N, 8.1120° E), Ebonyi Central (Ikwo; 6.0693° N, 8.1994° E) and Ebonyi South (Afikpo; 5.8895° N, 7.9538° E)) all in Ebonyi State. Ebonyi State is located in the Southern part of Nigeria. It is inhabited and populated primarily by the Igbo. The geographical coordinate of Ebonyi State is 6°15'N 8°05'E. Majority of Ebonyians are predominantly farmers.

2. 2. Methods

2. 2. 1. Collection and Processing of Soil Samples

The rice field soil samples were obtained from the plow layer (0 – 20 cm) of rice fields within the three agroecological zones of Ebonyi State: Ebonyi North, Ebonyi Central and Ebonyi South zones, using soil auger and transferred into sample opaque polythene bags. These were transferred to Department of Crop Science and Land Scape Management Screen House, Ebonyi State University, Abakaliki, Nigeria.

2. 2. 2. Experimental Design

For each plot, six bulk sub-samples was randomly collected and the sub–samples was homogenized to give one composite sample per plot. The soil was air-dried and stored at room temperature until the commencement of the experiment. Rice seeds (*Oryza sativa*) of four different accessions (Faro 44, Faro 52, Faro 59 and Faro 61) were obtained from Biotechnology Research and Development Centre, Ebonyi State University, Nigeria. The rice seeds were germinated in nursery and transplanted in a Screen house in different pots. Each Oryza accession was represented by three pots which each contained five seedlings and the pots were arranged in a randomized block design. Some pots were left unplanted (control). The pots were watered daily to maintain approximately 3 cm water overlying the soil.

2. 2. 3. Effect of plant growth stages, Soil samples and Rice Accessions on the microbial community composition

To determine the effect of plant growth stages on the culturable microbial community composition, this was done by monitoring rice plant height weekly. Four sampling time points were selected corresponding to stage 1 (day 34, early vegetative), stage 2 (day 52, late vegetative), stage 3 (day 62, reproductive), and stage 4 (day 90, maturity). Soil (planted pots), and bulksoil (unplanted pots) was collected aseptically using sterilized spatula. Plants were extracted from the pots and shaken to remove large soil aggregates and adhering soil remaining attached on the roots was considered to be rhizosphere soil and was sampled using a sterile spatula. Samples were immediately transferred to the laboratory for further analysis. To investigate how soil variation/sample might affect the microbial communities, plant harvested from the different soil samples were assayed as mentioned above, while the relationship between the different rice accessions and the microbial communities was equally sampled after incubation for 42 days in the Screen house.

2. 2. 4. Media preparation

All media used which included, Nutrient agar, MacConkey agar; Peptone water broth, Simmon Citrate agar etc were prepared following the manufacturer's instructions.

2. 2. 5. Isolation and identification of rhizosphere microbes isolated from rice plants

Nutrient agar, MacConkey agar and Simmons Citrate agar were employed for the isolation of bacteria for identification. Nutrient agar was used as a basic/or primary growth media for all microorganisms present on the sample, and MacConkey agar was used to isolate lactose fermenting gram-negative bacteria. All plates were incubated at 37 °C for 24 hours. Identification of bacteria isolates was based on the standard culture, morphological and biochemical methods (Cheesbrough, 2006).

2. 2. 6. Determination of aerobic plate count

Standard plate count method was used to determine the total aerobic colony count of the samples. Only plates with moderate growth were counted. The average microbial loads of the samples obtained from the different rhizospheric soil samples were expressed as colony forming units per Milliliter (Cfu/ml).

$$Cfu = \frac{N}{ND}$$

where: N = Number of colonies, V = Volume of sample transferred (0.5 ml), D = Dilution factor (10^{-5})

2. 3. Biochemical Identification Tests

Oxidase test, Catalase test, Citrate utilization test, Starch hydrolysis, Indole test, Voges – Proskaver (VP) test and Gram reaction test were carried out using standard methods described by Cheesbrough, 2006.

2. 4. Molecular Characterization of the Isolates

2. 4. 1. DNA Extraction from bacterial isolates randomly selected from planted and unplanted soil samples

Broth cultures were at different sampling points, randomly selected from planted and unplanted soil samples, incubated at 37 °C for 24 hours under continuous shaking to increase microbial population. One mililitre (1 ml) of bacterial culture was pipetted into sterile 1.5 ml eppendorf tubes. The tubes were centrifuged at 13,500 rpm for 5 mins and the supernatant was discarded while retaining the pellet undisturbed. A 100 µl of TE, 10 µl of lysosome and 10 µl of proteinase K was added into the tubes.

The tubes were incubated at 37 °C for 30 min, and 230 µl of cetyltrimethyl ammonium bromide (CTAB) (Hot 65 °C) was added to the tubes. A 100 µl of polyvinyl pyrrolidone (PVP) was also added. The tubes were vortexted and incubated at 65 °C for 30 minutes. Then, 450 µl of chloroform-isoamyl alcohol was added to the tubes and vortexed. The tubes were kept at room temperature for 10 minutes, and then centrifuged at 13,500 rpm for 5 minutes. The aqueous layer was then transferred into new tubes, and 100 µl of NaCl and 1 ml of 95 % alcohol were added into the tubes.

The tubes were stored at 20 °C overnight and centrifuged at 13,500 rpm for 5 min. Supernatant was discarded while the pellets were washed in 1 ml of 70 % alcohol. Supernatant were discarded while the tubes were air–dried by inverting over a paper towel.

Then 50 µl of sterile distilled water was added into each tube and DNA was recovered by placing the tubes in 65 °C water bath for 1 hour.

2. 4. 2. 16S rRNA gene amplification and RAPD

PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each of the 16SrRNA gene forward primer (16SF GTGCCAGCAGCCGCGCTAA) and reverse primer (16SR: AGACCCGGGAACGTATTCAC) and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8 µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94 °C for 5 minutes followed by a 30 cycles consisting of 94 °C for 30 seconds, 30 seconds annealing of primer at 56 °C and 72 °C for 1 minute 30 seconds and a final termination at 72°C for 10 minutes and chill at 4 °C.

2. 4. 3. Gel integrity

The integrity of the DNA and PCR amplification was checked on 1 % and 1.5 % agarose gel respectively. The buffer (1XTBE buffer) was prepared and subsequently used to prepare agarose gel.

2. 4. 4. Purification of amplified product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95 % ethanol were added to each about 40 µl PCR amplified product in a new sterile 1.5 µl tube eppendorf, mix thoroughly by vortexing and keep at -20 °C for at least 30 min. Centrifugation for 10 minutes at 13000 g and 4 °C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µl of 70 % ethanol and mix then centrifuge for 15 min at 7500 g and 4 °C. Again remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 minutes and then resuspended with 20 µl of sterile distilled water and kept in -20 °C prior to sequencing. The purified fragment was checked on a 1.5 % Agarose gel ran on a voltage of 110V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific.

2. 4. 5. DNA fingerprint and molecular genotyping of the organisms

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis

2. 5. Statistical Analysis

Statistical analyses were performed on all data that was generated using both one-way and two-way ANOVA procedure; using SAS statistical software version 9 at 0.05 probability level.

3. RESULTS

Table 1. Physico-Chemical properties of Soil Samples

Parameter	Zones		
	Ebonyi North	**Ebonyi Central**	**Ebonyi South**
% Sand	54.00	40.00	54.00
% Silt	25.00	30.00	28.00
% Clay	21.00	30.00	18.00
Texture	Sandy – Loam	Clay-loam	Sandy-loam
pH (H_2O)	5.40	5.80	5.30
P (mg/kg)	30.20	18.80	50.80
% N	1.40	0.070	0.182
% OC	1.37	0.85	2.31
% OM	2.36	1.46	3.98
Ca	8.00	7.40	3.20
Mg	4.80	2.00	1.20
K	0.184	0.297	0.200
Na	0.235	0.209	0.191

Key: OC = Organic carbon, OM = Organic matter

Table 2. Two-way ANOVA Table of Physicochemical Characteristics of the Soil Samples

	df	**Sum Sq**	**Mean Sq**	**F value**	**Pr (>F)**
Parameter	12	8943	745.2	21.780	3.61×10^{-09} ***
Location	2	41	20.6	0.602	0.557
Residual	21	719	34.2		
Significant codes:	0 '***'	0.001 '**'	0.01 '*'	0.05 '.'	0.1 ' ' 1

3. 1. Microbial Community structure between planted and bulk (unplanted) soil samples

The influence of the soil, rice plant and growth stages on the microbial community structure was determined by colony counting method using colony counting machine. The result revealed that microbial species were more abundant in rhizosphere (planted pots) than in check soil (unplanted pots). The highest microbial load was recorded in sampling stage (Stage 1; 32 Days After Planting, DAP) $15.8 \times 10 \pm 3.3 \times 10^6\ cfu/ml$ in planted against $10.7 \times 10 \pm 2.0 \times 10^6\ cfu/ml$ in unplanted while the lowest values were obtained on sampling stage 4 (90 DAP) which recorded $13.5 \times 10 \pm 3.5 \times 10^6\ cfu/ml$ in planted and $8.1 \times 10 \pm 0.7 \times 10^6\ cfu/ml$ in unplanted (Table 3). The microbial community structure of both planted and unplanted soil samples from the four data points showed that there was abundance of microorganisms in the planted compared to the unplanted samples. The two-way ANOVA (Table 4) showed significant difference in the microbial population across accessions and across days after planting but showed no significant increase across location.

Table 3. Microbial community structure as influence by rice plants.

Flooding period	Sample Type	Microbial load (cfu/ml)
1	Planted	$15.8 \times 10 \pm 3.3 \times 10^6$
	Unplanted	$10.7 \times 10 \pm 2.0 \times 10^6$
2	Planted	$14.9 \times 10 \pm 3.4 \times 10^6$
	Unplanted	$10.6 \times 10 \pm 1.0 \times 10^6$
3	Planted	$14.2 \times 10 \pm 3.5 \times 10^6$
	Unplanted	$8.6 \times 10 \pm 30.8 \times 10^6$
4	Planted	$13.5 \times 10 \pm 3.5 \times 10^6$
	Unplanted	$8.1 \times 10 \pm 0.7 \times 10^6$

Table 4. Two-way ANOVA on Impact of rice accession on microbial community structure across locations.

Parameter	df	Sum Sq	Mean Sq	F value	Pr (>F)
DAP	1	1.437×0^{14}	1.437×10^{14}	19.065	2.18×10^{-05} ***
Accessions	4	1.18×10^{15}	2.955×10^{14}	39.201	$< 2\times10^{-16}$ ***
DAP:Accessions	4	6.966×10^{11}	1.742×10^{11}	0.022	0.999

Locations	2	3.086×10^{13}	1.542×10^{13}	2.046	0.132
Residuals	172	1.297×10^{15}	7.539×10^{12}		
Significant codes:	0 '***'	0.001 '**'	0.01 '*'	0.05 '.'	0.1 ' ' 1

3. 2. Microbial populations in the rhizosphere of the rice accessions

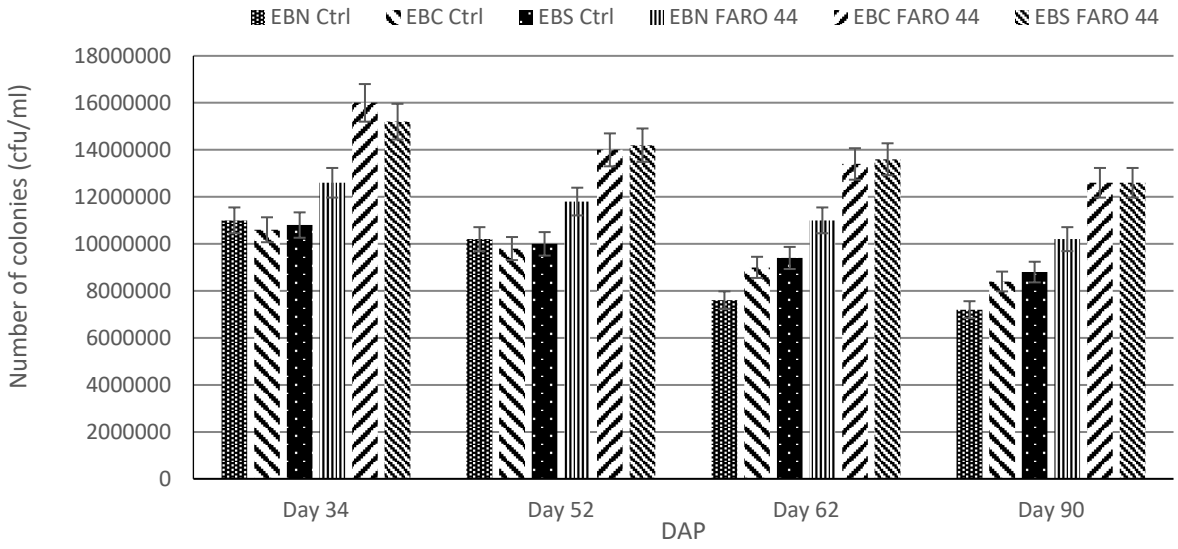

Figure 2. Microbial Population in Faro 44 Root Rhizosphere.

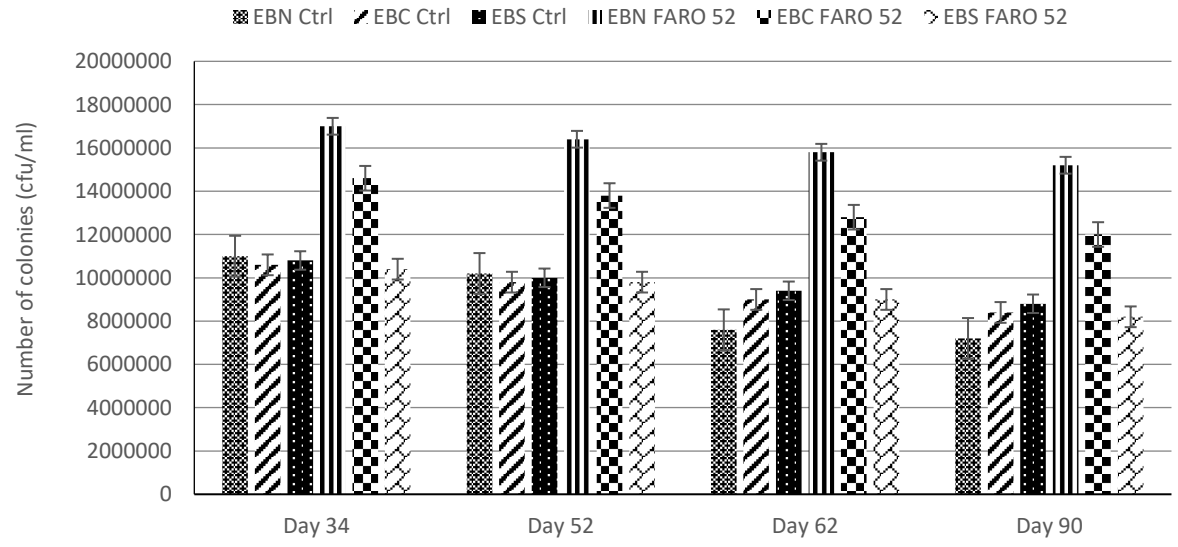

Figure 3. Microbial Population in Faro 52 Root Rhizosphere

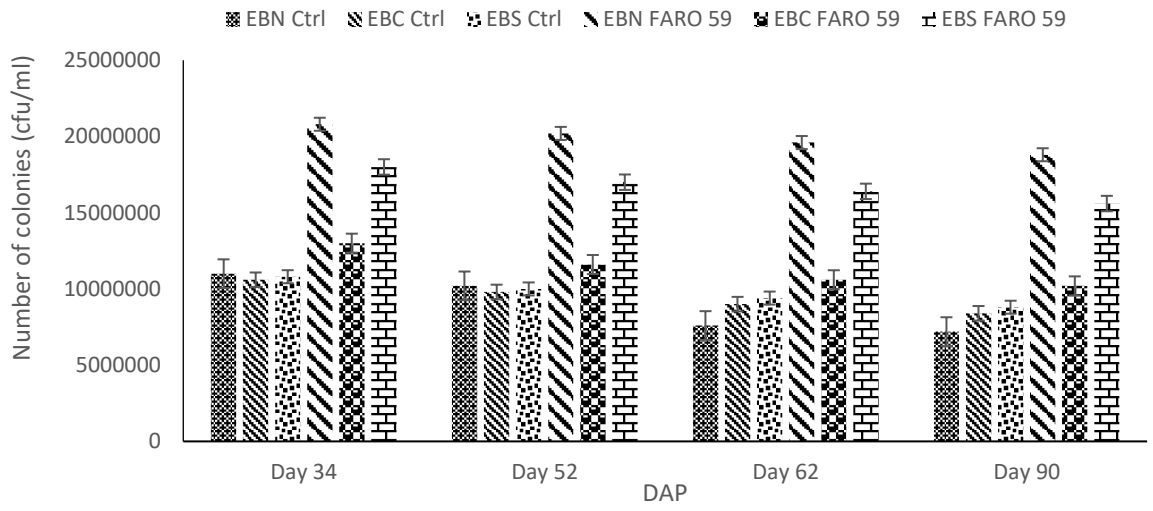

Figure 4. Microbial Population in Faro 59 Root Rhizosphere.

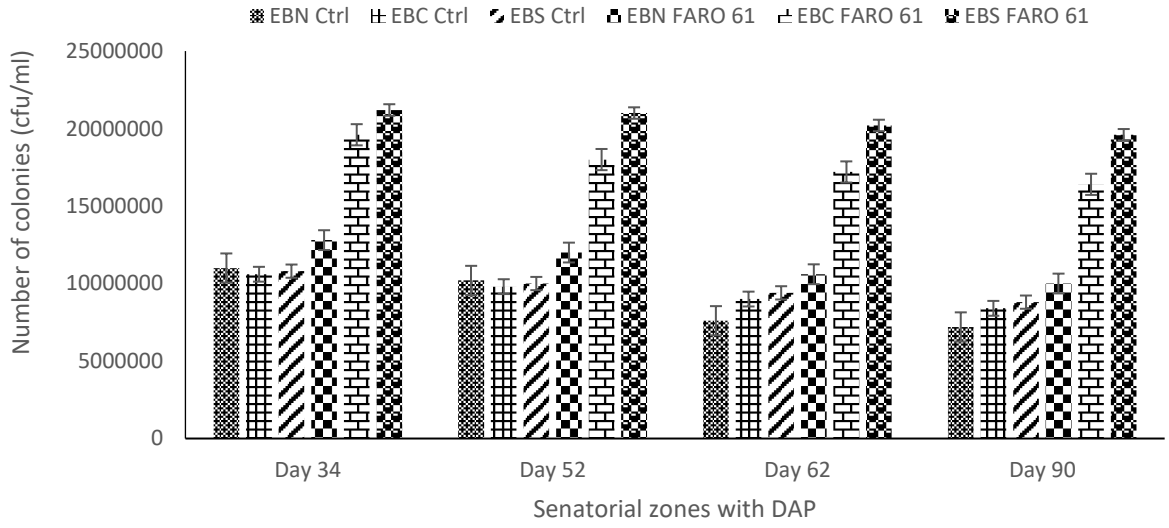

Figure 5. Microbial Population in Faro 61 Root Rhizosphere.

Table 5. The rice plant growth performances across the three zones of Ebonyi State on 90 DAP

Zone (Soil samples)	Faro 44 Height (cm)	Faro 52 Height (cm)	Faro 59 Height (cm)	Faro 61 Height (cm)
North	53.50	55.52	73.00	61.50

Central	72.50	67.80	76.00	69.50
South	74.50	64.30	75.80	74.00

Table 6. Two-way ANOVA table showing effect of DAP, accessions and Location on plant height.

Parameter	Df	Sum Sq	Mean Sq	F value	Pr (>F)
DAP	1	48891	48891	1386.33	$< 2x10^{-16}$ ***
Accessions	3	2263	754	21.39	$7.81x10^{-12}$ ***
DAP:Accessions	3	417	139	3.09	0.0286 *
Locations	2	2061	1030	29.22	$1.17x10^{-11}$ ***
Residuals	173	6101	35		
Significant codes:	0 '***'	0.001 '**'	0.01 '*'	0.05 '.'	0.1 ' ' 1

3. 3. Isolation, Identification and Characterization of the Microbial Community in the Rhizosphere of Rice in Ebonyi State Paddy Soil

3. 3. 1. Microbiological identity of the isolates

Morphological and biochemical identification of the isolates showed that Ebonyi North, Ebonyi Central and Ebonyi South samples had 40%, 20% and 60% of the isolates suspected to be Bacillus species, respectively. However, other suspected species include 40% Brevibacillus species and 20% Candida species in Ebonyi North sample, 60% Burkholderia species and 20% Aeromonas. hydrophila /caviae species in Ebonyi Central and 40% A. hydrophila /caviae species in Ebonyi South rhizosphere. All the isolates were rod shaped while biochemically, as shown in Tables 7; majority (53.33 %) of the isolates were gram positive, all were positive to catalase, citrate and oxidase tests while 11 (73.33 %) were starch hydrolyzing. All (15) of the isolates were negative to methyl red test but 13 (86.67 %) were positive to VP test.

Table 7. Percentage of some biochemical characteristics observed across the soil samples from the suspected rhizobacteria

Characteristics	No of isolates	(%)
Gram positive	8	53.33
Gram negative	7	46.67
Starch hydrolysing	11	73.33

Vp test Methyl red	13 15	86.67 100

3. 4. Molecular Characterization of the Biofertilizer Isolates

3. 4. 1. Gel Result of Agarose gel resolution of PCR Amplicons using different Primers

The PCR amplification was done using three RAPD primers viz, OPB05, OPT05 and OPB03 and resolved on agarose gel independently. The gel result of OPB05 revealed nine polymorphic alleles and 22 distinctly visible bands across the bacterial isolates amplified. The result showed that the most abundant alleles across the isolates was 600 bp in size occurring in 5 of the isolates followed by the 400 bp allele which occurred in 4 isolates while the lowest occurring alleles includes 400 bp, 500 bp and 1300 bp alleles which occurred in only one isolate each (Plate 1.) Meanwhile, OPT05 primer amplification product showed the presence of 5 alleles ranging from 400 bp to 1300 bp in size with 12 bands across the isolates. The 700 bp allele was most abundant being present in 6 (60 %) of the isolate while the 800 bp and 1300 bp alleles were least abundant occurring in only one isolate each as shown in Plate 2. Similarly, gel image of the DNA samples amplified with OPB03 revealed a total of 7 polymorphic alleles with a total of 31 amplified bands ranging from 500 bp to 1100 bp. The most abundant allele was the 700 bp allele which was present in 70 % of the isolates followed by 900 bp and 1 kb alleles present in 60 % of the isolates while the least abundant allele across the isolates was the 800 bp allele which occurred in 10 % of the isolates as shown in Plate 3.

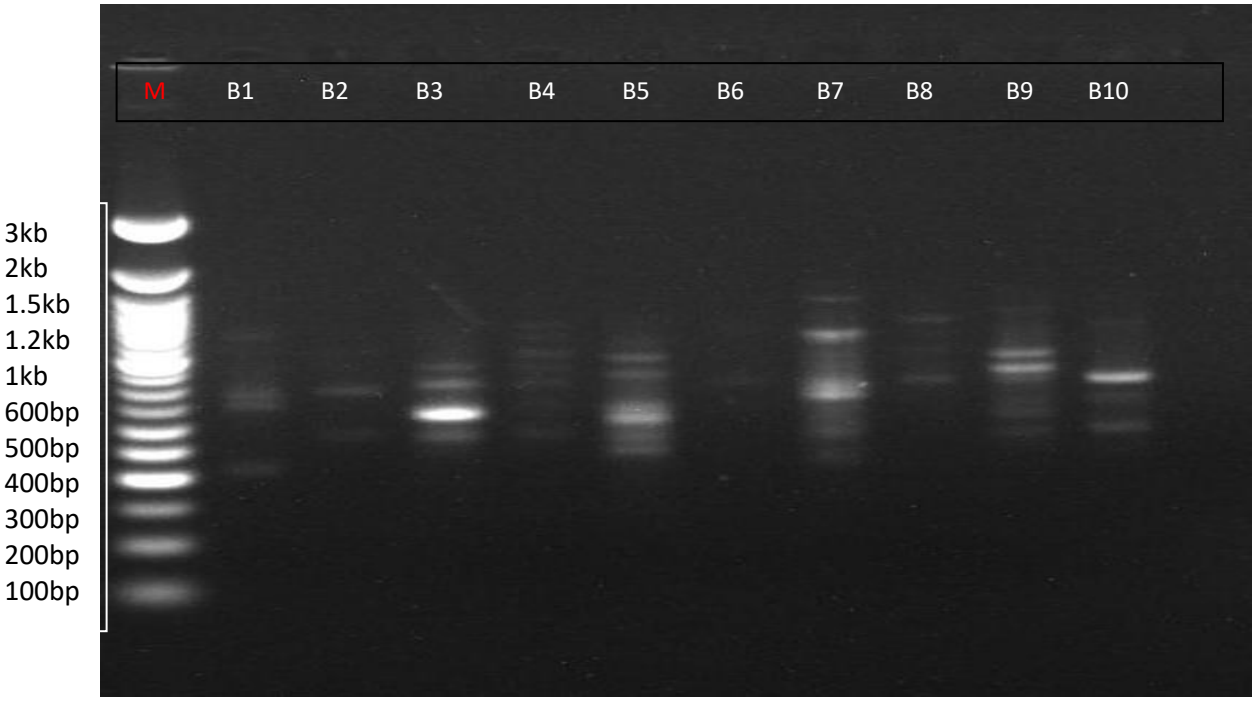

Plate 1. Gel resolution of Isolates DNA amplified with OPB05 RAPD Primer

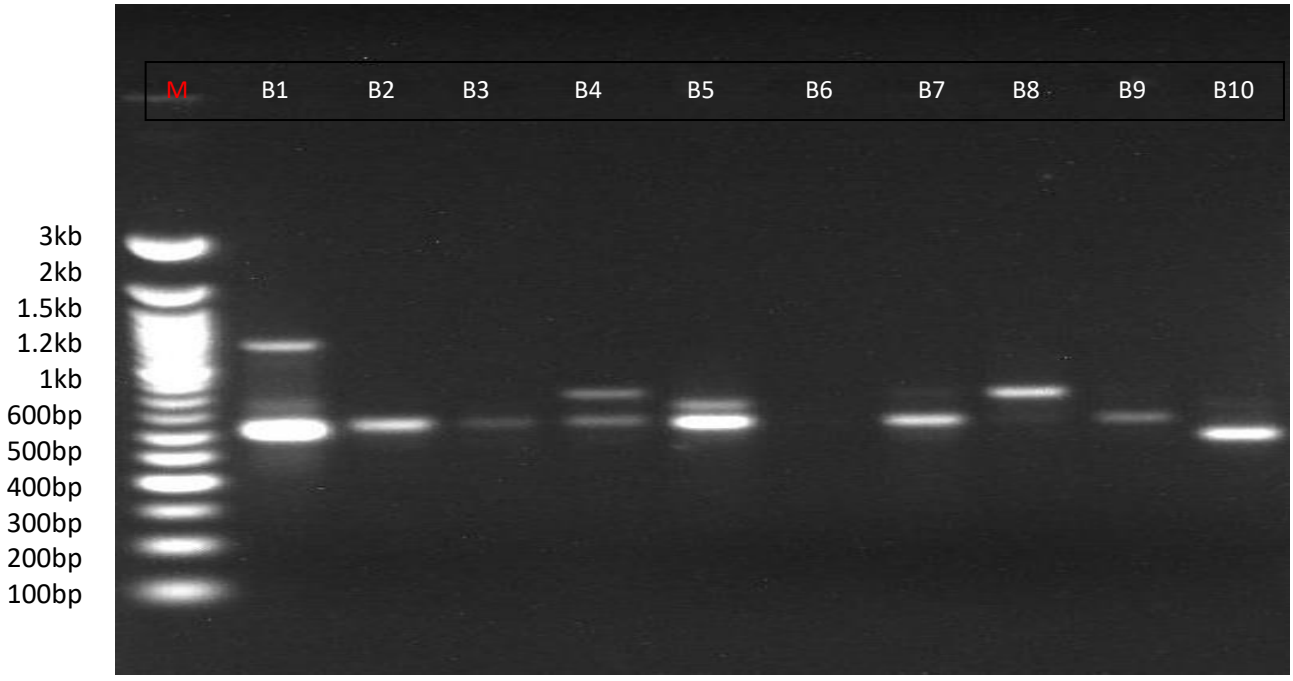

Plate 2. Gel resolution of Isolates DNA amplified with OPT05 RAPD Primer

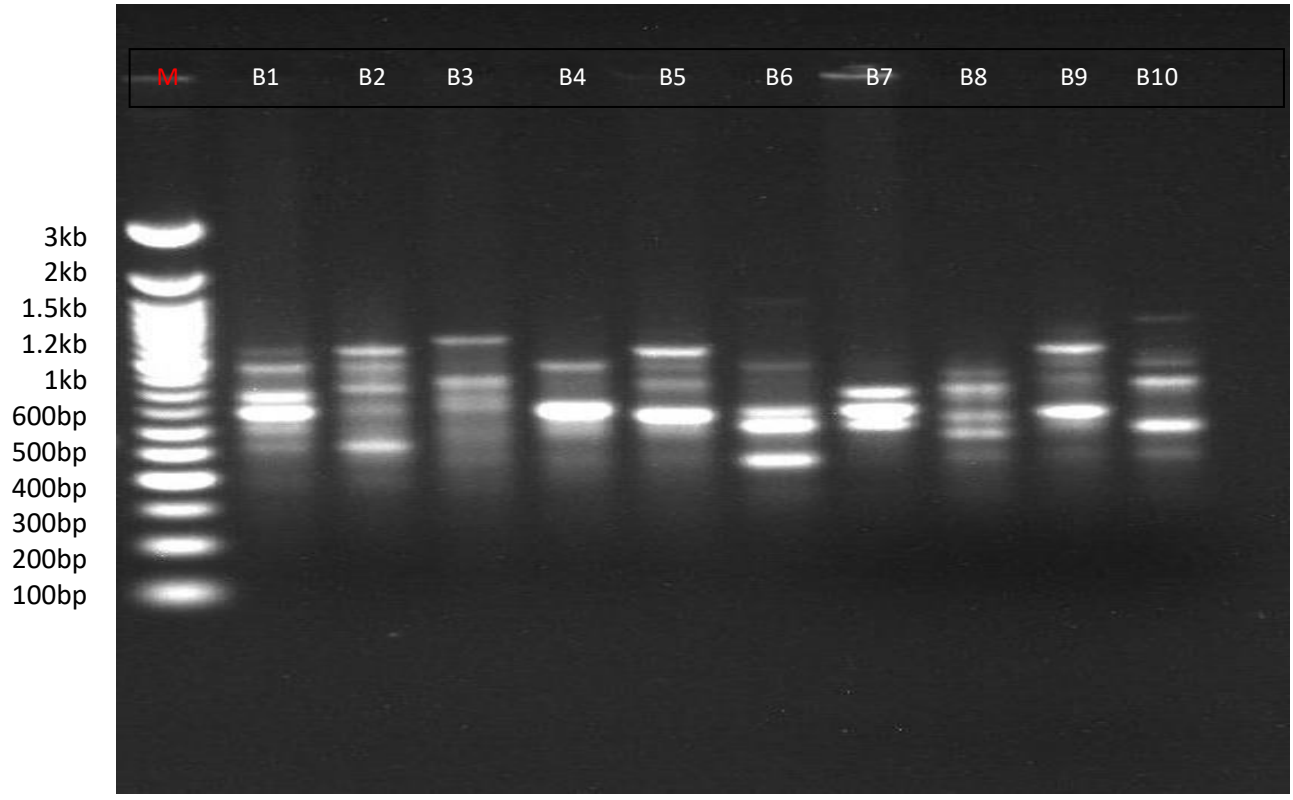

Plate 3. Gel resolution of Isolates DNA amplified with OPB03 RAPD Primer

3. 4. 2. In-silico Identification of Rhizobium Isolates

The result of the molecular identities of the bacterial isolates showed that isolate B1 was 88 % identical with *Aeromonas hydrophila* strain A210 16S with NCBI variety number MG062886. Meanwhile, the isolate with the highest similarity was B3 with 96 % similarity to *Aeromonas caviae* 16S, followed by B5 which was 93.90 % identical to *Pseudomonas otitidis* strain JK79 16S while B4 had lowest similarity with 72.20 % similarity to *Escherichia coli* strain 26561. The result is presented in Table 8, below.

NCBI Blast result

Table 8. Blast Result of DNA Sequences of the Bacterial Isolates

S/No.	Sample Code	Identified Organism	Pairwise Identity (%)	E Value	NCBI Variety Number
1	B1	*Aeromonas hydrophila* strain A210 16S	88.00	0	MG062886
2	B2	*Pseudomonas plecoglossicida* strain RJ39 16S	79.50	0	KJ818119
3	B3	*Aeromonas caviae* 16S	96.00	0	KF313551
`4	B4	*Escherichia coli* strain 26561	72.20	2.10E-08	CP027118
5	B5	*Pseudomonas otitidis* strain JK79 16S	93.90	0	MK578191
6	B6	*Enterobacter cloacae* strain ES-2 16S	89.40	0	MK537382
7	B7	*Serratia marcescens* strain AL105_R2A02 16S	86.40	0	KX928057
8	B8	*Cronobacter universalis* strain FC2941 16S	77.40	2.58E-42	MK396457
9	B9	*Lysinibacillus fusiformis* strain NBB1 16S	89.80	0	HQ256536
10	B10	*Aeromonas diversa* strain 2478-85 16S	90.10	0	NR_117303

3. 4. 3. Phylogenetic Relatedness among the isolates profiled for their molecular Characteristics

The result of the phylogenetic relatedness among the isolates revealed that isolates B1, B3 and B10 are closely related. It showed that 30 % of the isolates were *Aeromonas* species while 30 % belongs to the gamma proteobacteria species as shown in Figure 6 below.

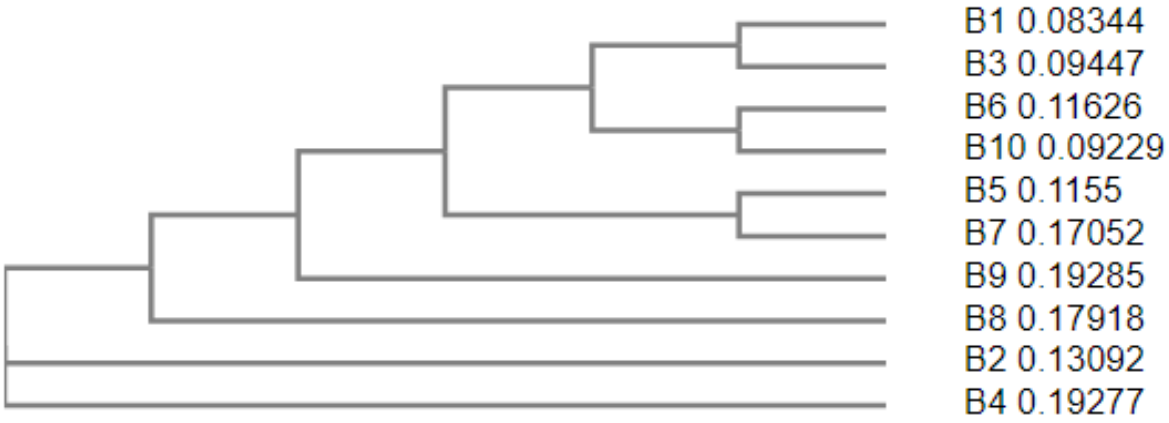

Figure 6. Phylogenetic relatedness among the different bacterial isolates

4. DISCUSSION

The present study was carried out to evaluate the presence of biofertilizer-microbes in the microbial community in the rhizosphere of rice plants from paddy soil in Ebonyi State, Nigeria as influenced by different Soil samples, Rice accessions at various growth stages. The ability of soils to remain biologically productive, maintain biological diversity, maintain environmental quality and enhance plant and animal health is vital in assessing soil health (Onweremadu and Oti, 2005). Hence, the reason for ascertaining the soil physicochemical parameters. Soil characterization, especially as it concerns the elemental distributions in soil, gives useful information for assessing and monitoring the behavior and fertility status/ level of different soil samples. Good knowledge of soil properties aids in determining soil characteristics, quality and productivity.

Though the 2-way ANOVA result showed significant variation ($p<0.001$) among the physicochemical properties in the samples as expected, it however revealed that there was no significant difference in the physicochemical contents of the soil samples across the locations, hence $p>0.05$ (Table 2). This implies that there was no significant difference in the soil types of the three zones in Ebonyi State with regards to their physicochemical makeup. The predominance of sandyloam in Ebonyi State, as in table 1 above, is in line with the findings of Aizebeokhai et al, 2005, who had a similar report from a research carried out in Southwestern Nigeria, and reported that the sandyloam soil was inherited from the parent material of the study area. The total aerobic colony count was determined with the Standard Plate Count method.

The average microbial loads of the samples obtained from the different rhizosphere soil samples at different sampling time points were expressed as colony forming units per Mililitre (cfu/ml). Microbial community population was highest in the first sampling stage (34 DAP) in both planted and unplanted soil and least on last sampling stage (stage four, 90DAP); this agrees with Di Cello et al, (1997), who stated that microorganisms are attracted and maintained at rhizodeposition pools of plant rhizosphere microhabitats. The result further revealed that the microbial community structure of planted soil pots had more microbes in abundance than the unplanted soil samples across the four data/sampling points. The bacterial abundance were on average less than twofold higher in rhizoshere (planted pots) than in check/or bulk soil

(unplanted pots). Planted pots were compared to check soil/or unplanted rice soil microcosm to control for changes in the community resulting from flooding and fertilization like sequential enrichment of microbes capable of exploiting diversity of electron acceptors (Shretha *et al.*, 2007). The significant variation observed in the microbial load between growth stage 1 and growth stage 4 is in agreement with Shretha et al., 2007, who reported that; because of the biotic and abiotic interactions between the plant roots and the content of the soil pore water in the rhizosphere, microbial concentration tends to increase in the Rhizosphere, a portion/or areas in the soil influenced by the plant where organic matter is introduced through rhizodeposition and sloughed – off cells.

The decrease in Rhizospheric microbial load with increase in sampling time/growth stages is in line with Aulakh et al. (2003) and Wu et al. (2009) that described rhizodeposition by rice plants and showed that it enhances microbial activity in the rhizosphere compared with check/or bulk soil and this in turn, could be detrimental to the growth of some selected microbes while favouring another (Butler et al., 2001). Rhizodeposition occurs all through the growth stages of plants in the form of water soluble exudates, secretions, and plant dead cells, forming a solution (pore water) that contributes as a primary source of energy and nutrients for microbes (Tian *et al*., 2013), this could be attributed to the high microbial load variations observed within and across the soil samples and the growth stages. Sampling stages 1 – 4, further revealed that the microbial counts (microbial abundance) were observed to decrease as the study progressed with 90 DAP recording the lowest colony counts across the locations and amongst the rice accessions studied in planted pots, while the decreased in check/or unplanted pots was a staggered one.

There were relatively few temporal changes in the microbial community (microbial diversity) either in check/or bulk soil and rhizosphere: This agrees with findings of Breidenbach and Conrad, (2014) that reported a uniform bacterial composition in soil over the rice growing season. This decrease in microbial load observed across the growth stages and soil samples may also be attributed to lack of nutrients which was orchestrated by the exhaustion of previously available nutrients in the soil sample. Similar to this observation, Chiara *et al.* (2012) observed variation in microbial load in rice paddy soil and also reported a gradual but staggered decrease in relative microbial abundance (Colony count) in rice rhizosphere soil samples. The result's shift in the microbial community correlated with soil conditions and crop growth stage with regard to the crop period, the early growth stage, had more microbial load compared with the later days of plant growth.

The result further revealed a general decrease in microbial load as the different rice accessions were planted on the different soil samples from the three Agroecological zones of Ebonyi state. This agrees with (Di Cello et al, (1997), who reported that " All things being equal, the identity and quality of rhizodeposits changes from one plant to another in a manner that it attracts a particular set of microbes to the rhizosphere, hence giving them a specific and selective pressure to stimulate or suppress bacteria to compete and persist", a property that relies upon plants' age..

The result of phylogenetic analysis of this research work revealed that some of the isolates belong to the phylum of proteobacteria, a major phylum of Gram – negative bacteria, and a wide variety of genera responsible for nitrogen fixation. This suggests that the presence of these bacteria may have been influenced by the rice plant accessions and in turn, contributed to the growth of the rice plants. However, the two-way ANOVA (Table 4) showed significant difference in the microbial population across accessions and across days after planting but

showed no significant increase across location. This implies that the decline in microbial population across the planting period may have been influenced by accessions but not by location. The general and gradual decrease in microbial load across growth stages and soil samples could again, be attributed to nutrient exhaustion with time, increased but differed chemical exudates secreted by plants which exert a selective microbial stimulation (Buir and Caessar, 1984, Miller, 1990) that varies in function of time due to plant age.Hence, as the growth stage advanced, the more adapted microorganisms at different growth stages may be favoured while the rest becomes less active and finally phased off. Di Cello *et al*, (1997) found that *Burkholderia cepacia* population associated with Maize roots decreased significantly during plant development, as we have just seen in this research result. Shibagaki et al, 2006, and Bernhard et al., 2005) reported that; to maintain adequate water depth in most of the crop year in flooded rice, the agricultural system has been characterized as being aquatic. Compared to other aquatic environments, like swamps, lakes and ponds, the condition in the environment of flooded rice field are relatively unstable because of physical, chemical and biological characteristics that differs according to current agricultural practices and water supplies, hence, the varying physical and chemical properties in such an environment could support the growth of microorganisms with a wide array of metabolic plasticity, equipping them with the arsenal to quickly adapt to changing environmental conditions.

Thus, the rice ecosystem may be a very good habitat for microorganisms adapted to fluctuating nutritional levels and oxygen and light availability hence, these could further support the presence of bacteria in the *Bacillus* and *Brevibacillus species*, *Aeromonas species* and the gamma–proteobacteria in the result of this research work. In line with these findings, Seiphepo et al. (2013) reported the abundance of Bacillus species in rice field sample and suggested that it is important in nitrogen fixation in the roots of rice and soil which enables the rice grow. To support the role of beneficial microorganisms in rice production, Seiphepo *et al.* (2013) stated that the use of beneficial soil microorganisms to help in improving crop production need the selection of rhizosphere-competent microorganisms that can promote plant growth. They reported that bacterial fertilizers supply the plant with nutrients and that a significant reduction in the use of nitrogen-fertilizer could be achieved if biological nitrogen fixation is made available to crop plants. In line with microbial communities obtained in this study, previously, researchers have some beneficial bacteria promote plant growth directly, that is, in the absence of pathogens.

Others do this indirectly by protecting the plant against soil-borne diseases. *Bacillus*, *Burkholderia*, *Aeromonas* and *Pseudomonas* species are some of the commonly isolated endophytes bacterial species from the rhizosphere (Babalola, 2010; Babalola and Akindolire, 2011). *Bacilli* were the dominant bacteria found in Ebonyi South and North and this correlate with other findings from rhizosphere studies (Gaur et al., 2004; Babalola and Akindolire, 2011). The dominance of *Bacillus* species may be due to the presence of endospores that can withstand extreme environments, hence very good for climate smart agriculture (Seiphepo e*t al.*, 2013).

This is because *Bacillus* can sporulate and so it can be formulated as a dry powder. Bacillus spp. is known for phosphate solubilization phytohormone production and siderophore production (Ode, 2008; Idris et al., 2009; Zhang et al., 2009). Also, Seiphepo et al. (2013) reported the presence of *A. hydrophila/caviae* among bacterial isolates from rice rhizosphere and stated that *Aeromonas* is an example of bacteria that produce siderophore. However, molecular identification of some of the isolates showed that 30 % of the isolates were *Aeromonas* species, 20 % of them was shown to be *Pseudomonas* species while *Lysinibacillus*

fusiformis, *Cronobacter universalis*, *Serratia marcescens*, *Enterobacter cloacae* and *Escherichia coli* made up the rest (Table 9). Phylogenetic analysis result revealed that some of the isolates belong to the phylum of Proteobacteria, a major phyum of Gram-negative bacteria, and a wide variety of genera, such as *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, *Yersinia*, *Legionellales*, and many others, including free-living (nonparasitic) bacteria many of which are responsible for nitrogen fixation.

This suggests that these bacteria may have contributed to the growth of rice plants. Meanwhile, the result showed that plant height increased as the study progressed. It was shown that most of the different rice accessions planted on samples from the three Agroecological zones of Ebonyi State performed better in Ebonyi South and Central zones compared to Ebonyi North zone. This varying growth performance can be attributed to the interaction between the indigenous rhizosphere bacterial population and the root system of the plant since phenotypic expression of certain genes is affected by gene-environment interaction. Also, the cluster analysis showed that 50 % of the isolates were *Aeromonas* species and were closely linked together, while about 30 % were classified as gamma proteobacteria, which according to Boden et al. (2017), are known to function in nitrogen and sulphur fixation such as *Azotobacter* species and *Pseudomonas* species. Ebonyi Central and South samples recorded higher percentage of the sulphur and nitrogen fixing bacteria in the family of the gamma proteobacteria. This can be attributed to the reason why rice accessions planted in Ebonyi South and Central recorded more height than that of Ebonyi North.

5. CONCLUSION

Soil microorganisms have long been recognized as major players in nutrient recycling, absorption and utilization in plant. This study has revealed that the indigenous microorganisms native to a particular location contributes significantly to the performance of a particular rice accessions planted on the soil. This was evident in the differential performance of each of the four rice accessions used in this study even under the same greenhouse condition. This suggests that it is best for each region to cultivate more of the rice accessions that performs best under the prevailing soil condition as informed by the indigenous microbial communities living and interaction with the plant roots thereby enhancing their optimum nutrient uptake; a microbial service that qualifies them as good candidates for biofertilizer production.

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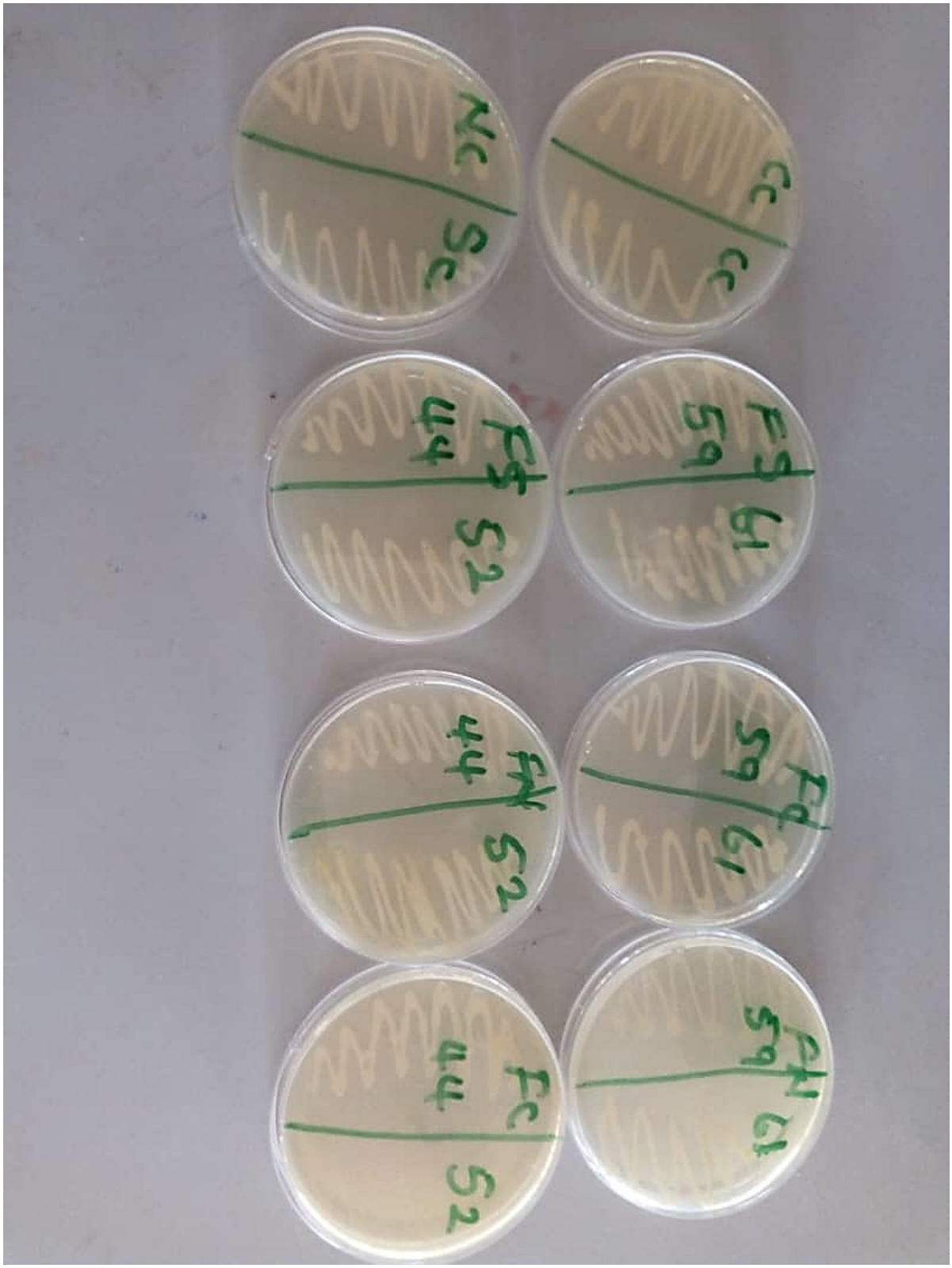
APPENDIX

The field and Laboratory analysis stages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