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Distinct Bacterial Community Composition in Rice Paddy Soil of Ebonyi State, Nigeria: Elucidation of Community Structure and Soil Physicochemical Properties

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Abstract

This study investigated the bacterial communities associated with different cultivars of rice from different flooded soil in Ebonyi State, Nigeria using molecular techniques as well as the physicochemical and pore water ion concentrations of different rice fields. Physicochemical parameters of sampled sites were determined using standard methods, while the pore water was analyzed using high performance Liquid chromatography and Ion chromatography. The PCR amplification was done using three RAPD primers namely, OPB05, OPT05 and OPB03 and resolved on agarose gel independently. The physicochemical parameters revealed the presence of sand, silt, clay, sandy - loam and clay – loam which were predominant in the area investigated, pH was slightly acidic, while there were presence of inorganic and inorganic elements.The amplified gel result of the three RAPD primers revealed that 9, 5, 7 polymorphic alleles and 22, 12, 31 distinctly visible bands across the bacterial isolates respectively with molecular base pairs ranging from 400bp-1300bp. Molecular identities of the bacterial isolates showed that *Aeromonas hydrophila* strain A210 16S, *Pseudomonas plecoglossicida* strain RJ39 16S, *Aeromonas caviae* 16S, *Escherichia coli* strain 26561, *Pseudomonas otitidis* strain JK79 16S, *Enterobacter cloacae* strain ES-2 16S, *Serratia marcescens* NBB1 16S and *Aeromonas diversa* strain 2478-85 16S were associated with the roots of different *Oryza* spp. Phylogenetic relatedness among the isolates revealed that isolates B1, B3 and B10 are closely related. It showed that 90% of the isolates mainly belong to the gamma proteobacteria, while 10% belong to the firmicutes. The study has given insight and understanding of the bacterial community composition of rice paddy soil in Ebonyi State.

Keywords: Bacteria, Flooded soil, Oryza spp, PCR
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Introduction

Rice (*Oryza sativa*) is one of the most important food crops in the world, nourishing approximately 50 % of the population and directly providing 20 % of human calorie intake (Zeigler and Barclay, 2008). Asia is the largest producer and consumer of rice (Khush, 2005; Sellamuthu *et al.*, 2011). In 2009, Nigeria was ranked 12th in the world's list of rice-consuming countries, third in Africa and first in West Africa, as producers of rice (FAO, 2011).

However, Nigerian rice production does not meet current demand or have the capacity to cope with an expanding population. Production is also

suggested to be declining due to effects of climate change particularly through drought, heat, flooding and pests and diseases (Rosenzweig et al., 2000)

Rice differs from most crops in that it is typically cultivated in flooded soil, resulting in oxic and anoxic zones within the rice rhizosphere that select for specific physiological groups of microorganisms with aerobic, anaerobic, or facultative metabolism (Brune *et al.,* 2000). Methanogenesis in the rhizosphere and bulk soil of rice fields results in high methane (CH_4) production, with rice agriculture currently contributing ~10 % of the global CH_4 budget (Conrad, 2009). The primary substrates for methanogens are acetate or H_2 + CO_2 produced from the breakdown of complex carbon by the microbial community, including fermenters and acetogens (McInerney *et al*., 2008). Approximately 60 % of CH_4 produced in rice fields originates from root exudates or decaying root material (Watanabe *et al.*1999).

Soil, being a natural and non-renewable fixed asset, found on the surface of the earth, supports human existence, sustains biological productivity, diversity and maintains environmental quality (Onweremadu and Oti, 2005). Having a sound knowledge of soil's complex and dynamic nature is of immense importance in nurturing healthy plant and crop yield improvement. When elemental distributions in soil are characterized, it provides vital information for assessing and monitoring the behavior and fertility level of these soils. The fertility level further helps in deciding/ or choosing which soil is good for agricultural and non-agricultural purposes. Those with good fertility level and good for agriculture further determine the plant type that thrives in it.

There has been interest in the properties of soil-associated microbiota driven by the relative ease of quantifying microbes based on metagenomic and next generation sequencing (Lundberg et al., 2012. Such an approach has led to a significantly better understanding of community structure and function in the rhizosphere in the last decade. A novel and recommended approach is to exploit the role of soil microbial communities for sustainable and healthy crop production, while preserving the biosphere. Soil microorganisms play fundamental roles (microbial services) in agriculture usually by improving plant nutrition and health, as well as soil quality (Barea *et al.,* 2013, Lugtenberg., 2015). Accordingly, several strategies for a more effective exploitation of beneficial microbial services, as low-input biotechnology to help sustain environmentally friendly agro-technological practices have been, and are being, proposed.

Studies were carried out on microbial communities inhabiting the rice field ecosystem, for instance, the microbes within the rice interior, the rhizoplane and the rhizosphere (Edwards et al., 2015). Further studies also have investigated the rice phyllosphere microbial community by 16S rRNA pyrotag sequencing (Ren *et al.,* 2014) as well as endophytic and rhizospheric communities with metagenomic and metaproteomic approaches (Knief *et al.,* 2012). Experiments have identified the bacteria and archea (Hernández *et al.,* 2015) that consume plant-derived carbon in the rhizosphere. Also, specific functional groups of microorganisms, such as methanogens (Lee *et al.,* 2014), have been extensively analyzed in rice systems.

Knowledge of the native bacterial population, their characteristics, and identification is required for understanding the distribution and diversity of indigenous bacteria in the rhizosphere of rice plant in Ebonyi State which will lead to a boost in rice yield, keeping this in mind the study was planned to isolate and identify the native strains of indigenous bacteria from rice rhizosphere grown in different zones of Ebonyi using 16S rRNA sequence and also determine the physicochemical and pore water characteristics of the paddy soil.

Materials and Methods

Study Site

The area under study includes the three zones that make up Ebonyi State, viz: Ebonyi North, Ebonyi Central and Ebonyi South. Ebonyi State rests within longitude 7.30' and 8.30'E and latitude 5.40' and 6.45'N. The state was created on 1st of October 1996 from the former Abia and Enugu states, with Abakaliki as its capital. It is located in the southeastern region of Nigeria. It is bounded to the north by Benue State, to the west by Enugu State, to the east by Cross River State and to the south by Abia State.

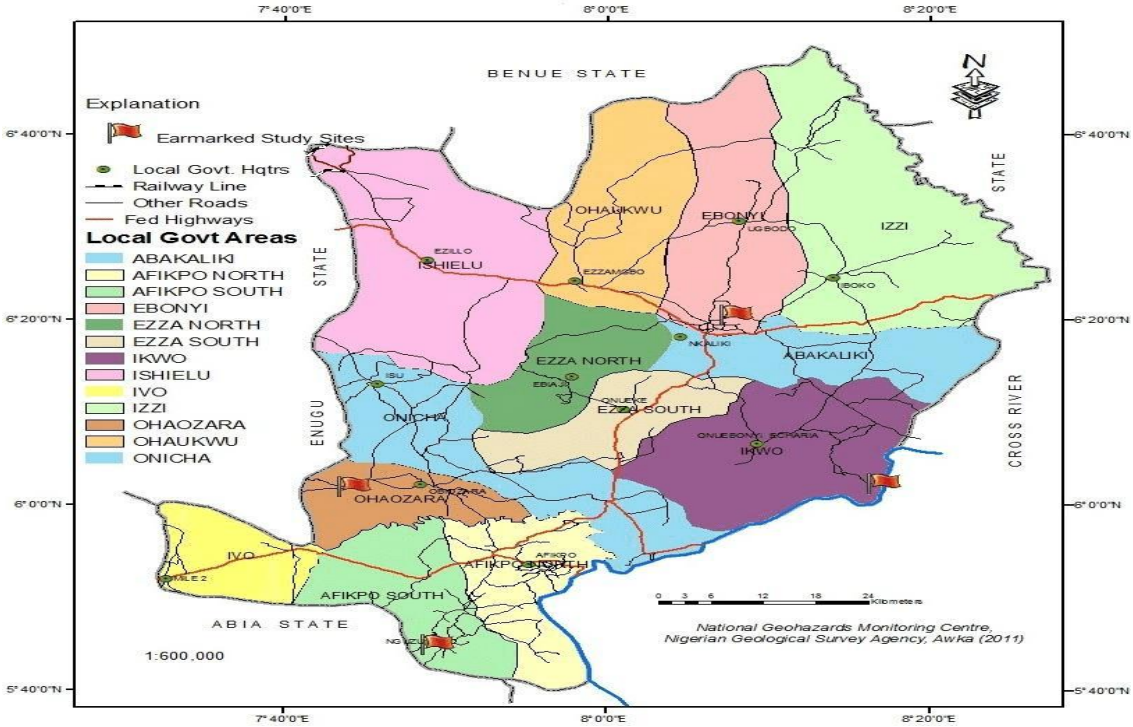

Figure 1: Map of Ebonyi State showing local government areas that made up of the three zones

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 zones of Ebonyi State, Nigeria: Ebonyi North, Ebonyi Central and Ebonyi South Zones which had been planted for at least 3 years. The soil samples were collected using stainless handheld shovel 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. For each plot, six bulk sub-samples were randomly collected and homogenized to give one composite sample per plot. The soil was air-dried, sieved, and stored at room temperature until the commencement of the experiment. The soil basic characteristics such as pH, soil organic carbon, total nitrogen, available phosphorous, total phosphorous were determined using standard methods (AOAC, 1965).

Collection and planting of rice samples and incubation study

Rice seeds (*Oryza sativa*) of four different accessions (Faro 44, Faro 52, Faro 59, and Faro 61) were obtained from Biotechnology Research Centre, Ebonyi State University, Abakaliki, Nigeria. The rice seeds were germinated in nursery and transplanted in a green house in different pots each containing different soil samples obtained from three (3) different locations of the State; namely; Ikwo, Abakaliki and Afikpo. 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). Prior to the establishment of

microcosms, soil was sieved through a 0.2mm stainless steel screen and 2.0 kg was introduced into opaque plastic pots (10 cm height, 17.0 cm diameter). The pots were flooded with deionized water 1 week before planting. Fertilizers included Urea (CH_4N_2O, 45 gl^{-1}) as Nitrogen source, Phosphorus ($Na_2HPO_4{\cdot}2H_2O$, 17 gl^{-1}), Potassium (KCl, 50 gl^{-1}), and Magnesium ($MgSO_4{\cdot}7H_2O$, 2 gl^{-1}) were added. Also, phosphorus, potassium and magnesium solutions were added at a ratio of 10 ml kg^{-1} soil a day before planting, whereas 5 ml kg^{-1} of the urea were added twice: a day before planting and after 14 days of plant growth (Hantolo, 1995). The pots were watered daily to maintain approximately 3 cm water overlying the soil. The pots were incubated for 4 weeks in a greenhouse with a 12 hour light/dark cycle. Soils were sampled from both the rhizosphere and bulk soil at intervals for analysis of bacterial communities, namely rhizosphere soil of whole root system of the different rice accessions and bulk soil of the non-planted pots.

Pore water analysis

Soil samples (100g) collected from the planted and unplanted plots were centrifuged for 10 min at 20,000 *g* and 4 °C in 50 mL centrifuge tubes. The supernatant was filter sterilized using 0.2 µm acetate-free filters (GE Health care Life Science, Freiburg, Germany) and stored at -20 °C until analysis. Organic acids were analyzed with high performance liquid chromatography (HPLC; pumpS1000, oven S4110 (Sykam), Germany. Inorganic ions including chloride, nitrate, nitrite, phosphate, and sulfate were detected using ion chromatography (IC; pump S1121, sampler S5200 (Sykam); Bak *et al.,* 1991).

Collection of rhizosphere soil and recovery of rhizobacteria

The recovery of rhizospheric soil was done by cutting the sides of the pots, the rice seedlings was lifted and shaken to remove soil loosely adhering to the roots and the attached soil on the roots were taken as rhizosphere soil. The sample was taken as rhizosphere soil. The unplanted pot containing soil treated in same manner as the planted pots were cut and the sides of the pots removed. The soil from these pots was stored as the bulk soil samples. The bacteria from the rhizosphere soil of rice plant and bulk sample were isolated using nutrient agar (Oxoid). 1g each soil samples in sterile flask with 9 ml water was agitated by vigorous shaking for 5 mins while the suspension was in motion, 10 ml was withdrawn and added to 90 ml sterile water in a screw cap flask and was shaken for I min, and 1o ml of the suspension was transferred to 90 ml sterile water blank. The process was repeated until dilution of 1: 1000ppm. One ml of the dilution was spread on nutrient agar surface and an inclined rotary motion of the plate. Plates were incubated at 37°C for 24 h

Morphological and Biochemical Characterization of Isolates

Phenotypic characterizations of pure bacterial cultures were performed according to Bergy's manual of determinative Bacteriology (Buchanan and Gibbons, 1974). Shape, Grams reaction, arrangement and presence of endospore, were observed with a 1000 x magnification microscope, while relevant biochemical characterization such as catalase production, oxidase production, citrate utilization, starch hydrolysis, gelatin hydrolysis,, methyl red, Voge-Proskauer test were carried out (Buchanan and Gibbons, 1974, Cheesbrough, 2007).

Molecular Characterization of the Isolates

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 milliliter (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, 10µl of lysosome and 10µl 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.

16S rRNA gene amplification and RAPD

PCR sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25mM $MgCl_2$, 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.

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. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60°C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 µl) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a hyper ladder1 that was ran alongside experimental samples in the gel.

Purification of amplified product

After gel integrity, the amplified fragments were ethanol purified to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3M and 240 µl of 95 % ethanol were added to each a40 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 -20oC 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.

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

Results

Physico-chemical Properties of Soil Samples from the three Zones in Ebonyi State

The result of the physico-chemical properties of the soil samples collected from rice field at different locationin Ebonyi State is presented in Table 2. It showed that the soil sample from

Ebonyi North and Ebonyi South had a higher sand fraction (54.00 % and 54.00 %) compared to the sample from Ebonyi Central (40.00 %). While, silt was higher in Ebonyi Central (30.00 %), both Ebonyi North and South samples had sandy-loam texture while Central was more of clay-loam in texture.

Ebonyi South recorded the highest Phosphorus, organic carbon, and organic matter contents (50.80 mg/kg, 2.31 % and 3.98 %) but Ebonyi North samples recorded the highest calcium and magnesium content (8.00 mg/kg and 4.80 mg/kg respectively).

The result of the 2-way analysis of variance showed significant variation ($p<0.001$) among the physicochemical properties in in sample. However, there was no significant difference in the physicochemical contents of the soil across the locations, hence $p> 0.05$ (Table 2).

Table 1: Physico-Chemical Properties of Soil Sample from the three Zones of the State.

	Zones		
Parameter	**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

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	3.61e-09 ***
Location	2	41	20.6	0.602	0.557
Residual	21	719	34.2		

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Concentration of Organic and Inorganic Ions

The organic and inorganic ion contents of the rice rhizosphere throughout the plant period in Ebonyi North, Central and South samples are shown in Tables 3-5 below.

Tables 3, 4 and 5 show the concentrations of organic and inorganic ions detected in the pore water of rhizosphere (planted) and bulk soil (unplanted) from Ebonyi North, Central and South soil respectively, at stages 1- 4. The result of the soil pore water analyses showed that acetate, lactate, formate, chloride, and propionate concentrations were significantly ($p < 0.05$) higher in the planted pots across the three senatorial zones compared to the unplanted pots. The concentrations of malate and nitrate were significantly ($p<0.05$) higher in unplanted pots across all zones than in planted pots. While sulfate concentration varied between planted and unplanted pots across the three zones, the variation was not significantly different.

The 2-way Anova result showed that there was significant difference in the ion content among the planted soil sample across the location but the unplanted soil showed no significant variation. Also, there was no significant difference in the ion content across the three locations profiles (Table 6).

Table 3: Concentrations of organic acids and inorganic ions detected in the pore water of rhizosphere (planted) and bulk soil (unplanted) from Ebonyi North Soil at stages 1-4

Ion	category	34 days	52 days	62 days	90 days
Acetate	Planted	0.44a	0.49a	0.49a	0.53a
	Unplanted	0.02b	0.01b	ND	0.01b
Lactate	Planted	0.96b	1.40a	1.16b	1.25ab
	Unplanted	0.16c	0.02e	0.04d	0.04d
Malate	Planted	69.24f	70.12e	102.5c	120.89b
	Unplanted	85.95d	101.30c	117.90b	147.13a
Formate	Planted	0.14b	0.10b	0.30a	0.39a
	Unplanted	0.03d	0.08c	0.05cd	0.04d
Chloride	Planted	12.69d	31.85a	29.05a	34.90a
	Unplanted	12.04d	14.48c	16.45bc	18.98b
Nitrate	Planted	0.02c	0.01c	0.04b	ND
	Unplanted	0.14a	0.05b	0.14a	0.24a
Propionate	Planted	0.06d	1.16a	0.38c	0.95b
	Unplanted	ND	ND	0.03d	ND
Sulfate	Planted	0.03a	0.02a	0.02a	0.03a
	Unplanted	0.02a	0.02a	0.03a	0.04a

(Values indicate the mean ion in millimolar concentrations. Values having the same alphabets are not different ANOVA, P =0.05). ND= Not detected

Table 4: Concentrations of organic acids and inorganic ions detected in the pore water of rhizosphere (planted) and bulk soil (unplanted) from Ebonyi Central Soil at stages 1-4

Ions	**category**	**34 days**	**52 days**	**62 days**	**90 days**
Acetate	Planted	0.44b	0.50a	0.48a	0.54a
	Unplanted	0.03c	0.01c	ND	0.01c
Lactate	Planted	0.98b	1.41a	1.18a	1.25a
	Unplanted	0.18c	0.02d	0.03d	0.04d
Malate	Planted	69.28c	70.18c	101.5b	121.89b
	Unplanted	86.95c	102.30b	116.90b	148.13a
Formate	Planted	0.13b	0.10b	0.31a	0.40a
	Unplanted	0.02c	0.09bc	0.05c	0.04c
Chloride	Planted	12.69c	30.95a	29.05a	34.90a
	Unplanted	12.05c	14.49bc	16.47b	19.10b
Nitrate	Planted	0.02c	0.01c	0.05b	ND
	Unplanted	0.13a	0.05b	0.13a	0.24a
Propionate	Planted	0.06c	1.17a	0.36b	0.96a
	Unplanted	ND	ND	0.03c	ND
Sulfate	Planted	0.03a	0.02a	0.02a	0.03a
	Unplanted	0.02a	0.02a	0.03a	0.05a

(Values indicate the mean ion in millimolar concentrations. Values having the same alphabets are not different ANOVA, P = 0.05). ND= Not detected

Table 5: concentrations of organic acids and inorganic ions detected in the pore water of rhizosphere (planted) and bulk soil (unplanted) from Ebonyi South Soil at stages 1-4

Ion	Category	34	52	62	90
Acetate	Planted	0.43a	0.48a	0.48a	0.51a
	Unplanted	0.02b	0.01b	ND	0.01b
Lactate	Planted	0.97b	1.41a	1.15ab	1.24a
	Unplanted	0.16c	0.01d	0.03d	0.04d
Malate	Planted	69.23c	70.13c	102.5b	121.89b
	Unplanted	85.97c	101.31c	118.90a	147.13a
Formate	Planted	0.14b	0.10b	0.30a	0.39a
	Unplanted	0.03d	0.09c	0.05c	0.04cd
Chloride	Planted	12.70c	31.85a	29.05ac	34.91a
	Unplanted	12.04c	14.48bc	16.46b	18.98b
Nitrate	Planted	0.02c	0.01c	0.05b	ND
	Unplanted	0.13a	0.05b	0.14a	0.23a
Propionate	Planted	0.07c	1.17a	0.38b	0.95b
	Unplanted	ND	ND	0.03c	ND
Sulfate	Planted	0.03a	0.02a	0.02a	0.03a
	Unplanted	0.02a	0.02a	0.03a	0.04a

(Values indicate the mean ion in millimolar concentrations. Values having the same alphabets are not different ANOVA, P =0.05). ND= Not detected

Table 6: Two-way Anova Table showing interaction in parameters between plant and unplanted soil samples acros locations.

Parameter	**Df**	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Planted	1	24.6	24.552	4.750	0.0319 *
Unplanted	1	3.8	3.771	0.730	0.3952
Planted:Unplanted	1	0.1	0.149	0.029	0.8656
Location	1	0.2	0.219	0.042	0.8374
Residuals	92	475.5	5.169		

Microbiological Identity of the isolates

Table 7, shows the morphological (such as shape and colour) which were used to separate the different colonies that were present on the nutrient agar plates and biochemical characteristics of the bacterial species isolated from rice rhizosphere. Morphologically, all the isolates were rod shaped, it also showed that majority (53.33 %) were gram positive and also positive to catalase, citrate and oxidase tests, while 11 (73.33 %) were starch hydrolyzing. Also all negative to methyl red test but 13 (86.67 %) were positive to VP test.

Table 7. Morphological, Physiological and Biochemical characteristics of Bacterial Isolates from Rhizopheric soil from the three zones of the state

	Culturable Rhizobacteria														
Characteristics	**NC**	**FN 44**	**FN52**	**FN59**	**FN61**	**CC**	**FC44**	**FC52**	**FC59**	**FC61**	**SC**	**FS44**	**FS52**	**FS59**	**FS61**
Gram Reaction	+	+	+	-	+	+	-	-	-	-	-	+	+	-	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate Utilization	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Starch Utilization	-	+	+	+	-	+	+	-	-	+	+	+	+	+	+
Indole	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Methyl Red	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskaver	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+
Endospore staining	+	+	+	_	+	+	_	_	_	_	_	+	+	_	+
Suspected Microbes	*Brevibacillus spp.*	*Bacillus spp*	*Bacillus spp*	*C. freundii*	*Brevibacillus spp.*	*Bacillus spp.*	*Burkholderia gladioli*	*Burkholderia cepacia*	*Burkholderia gladioli*	*A. Hydrophila /caviae*	*A. Hydrophila /caviae*	*Bacillus spp*	*Bacillus spp*	*A. Hydrophila /caviae*	*Bacillus spp*

- = negative, + = positive

Molecular characterization of the isolates

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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 (Fig. 2).

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 Fig. 3. 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 Fig. 4.

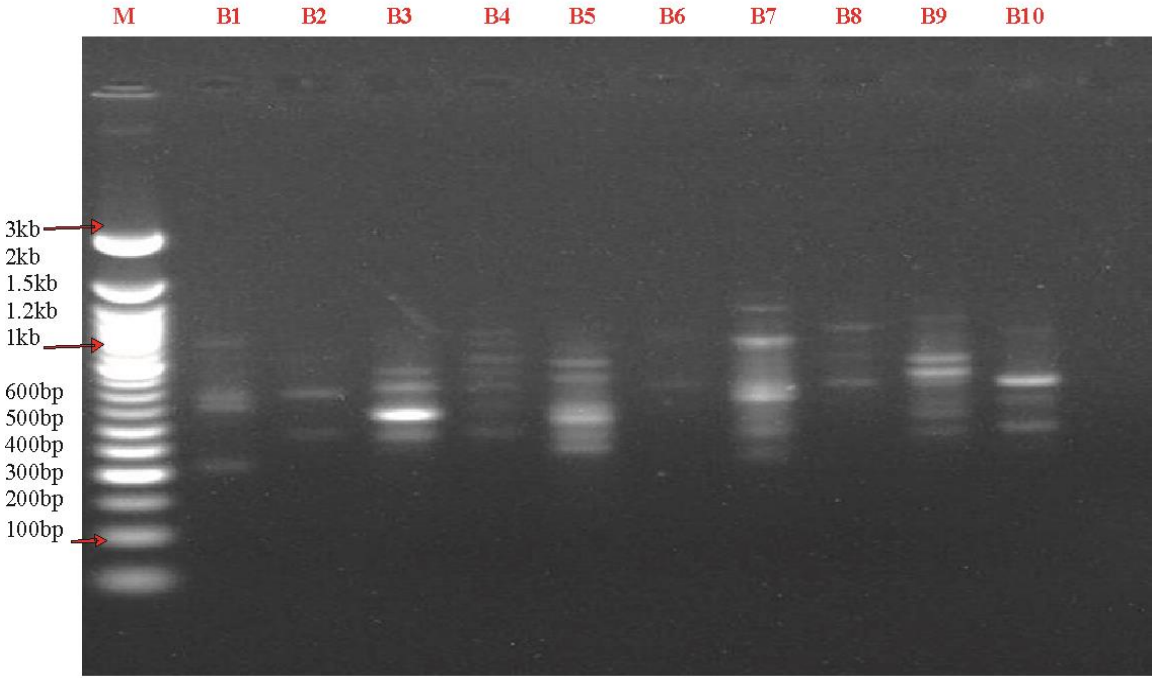

Fig. 2: Gel resolution of Isolates DNA amplified with OPB05 RAPD Primer
M represent I kb marker, B1 – B10 represents different isolates

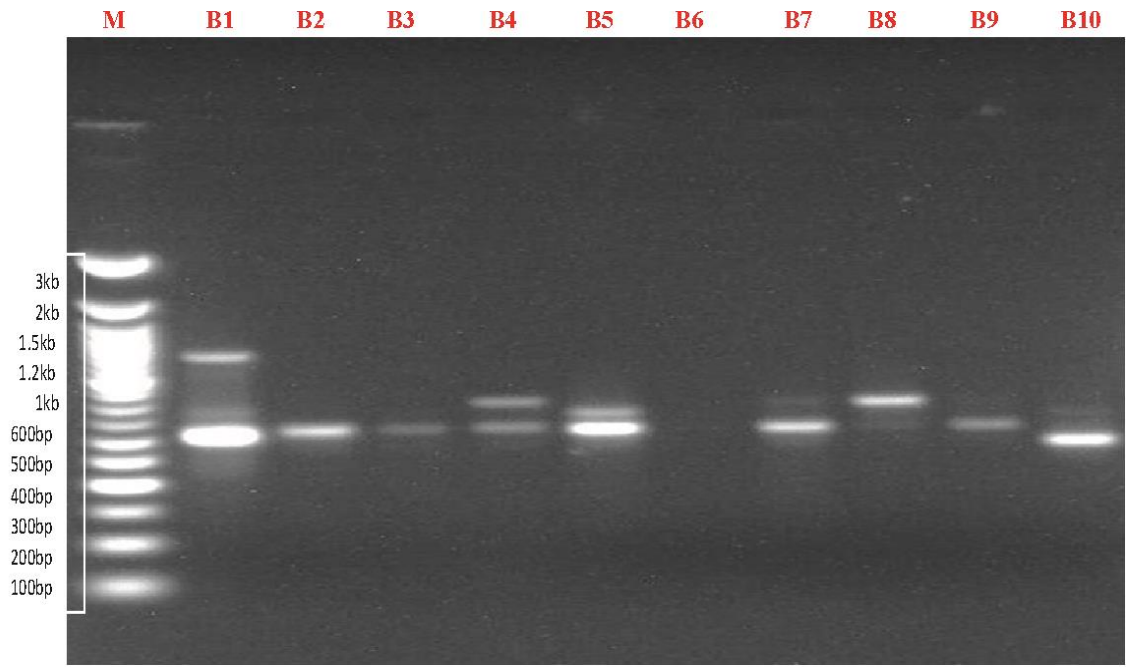

Fig. 3: Gel resolution of Isolates DNA amplified with OPT05 RAPD Primer
M represent I kb marker, B1 – B10 represents different isolates

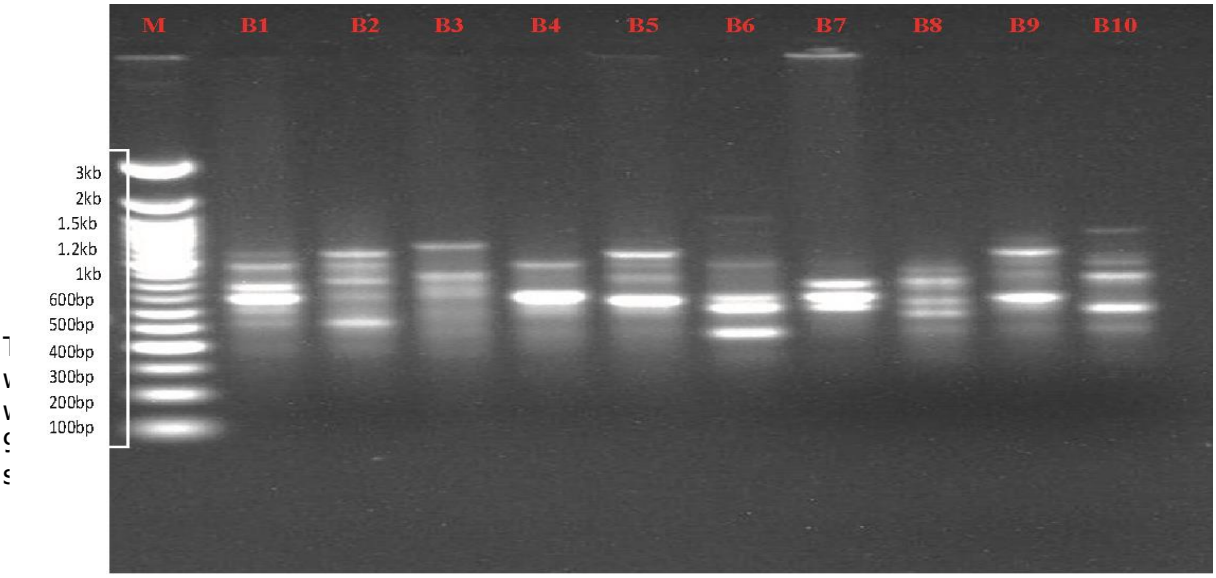

Fig. 4: Gel resolution of Isolates DNA amplified with OPB03 RAPD Primer
M represent I kb marker, B1 – B10 represents different isolates

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

S/No.	Sample Code	Identified Organism	Pairwise Identity	E Value	NCBI Accession 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	*Serratiamarcescens* 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

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 5 below.

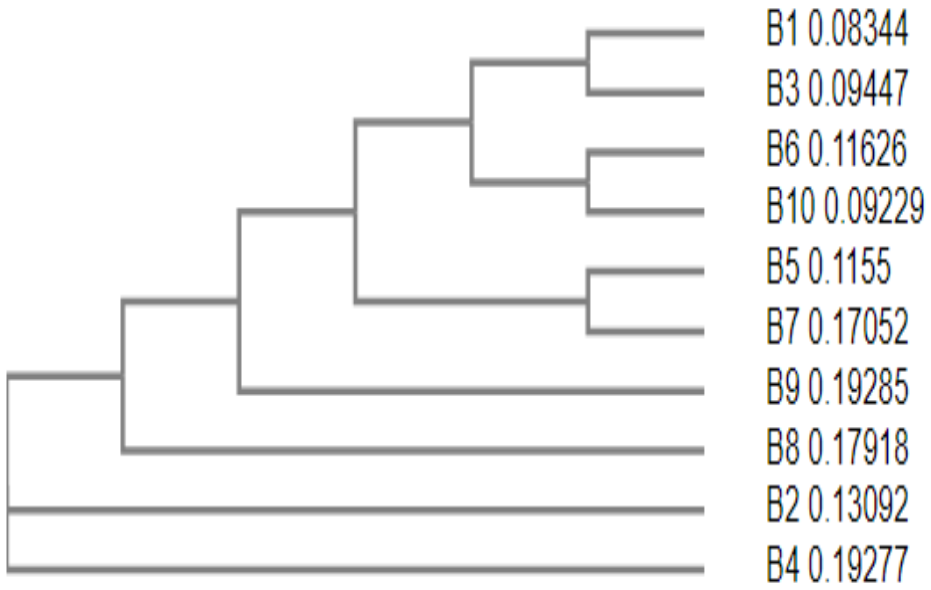

Fig 5: Phylogenetic relatedness between different bacterial isolates. Genetic distances were obtained by RAPD analysis with a primer

Discussion

For proper crop yield, it's vital to observe proper soil management to sustain agricultural production, keep up with the environmental health (Ashenafi *et al.,* 2010), and for general economic growth (Muche *et al.,* 2015). The ability of soils to retain biological productivity and diversity, maintain environmental quality and enhance plant and animal health is vital in assessing soil health (Onweremadu and Oti, 2005). Soil characterization, especially as it concerns the elemental distributions in soil, gives useful information for assessing and monitoring the behavior and fertility status of different soil samples. Good knowledge of soil properties aids in determining soil characteristics, quality and productivity.

The predominance of sandy loam soil in Ebonyi state is in line with findings of Aizebeokhai *et al* (2018), who had a similar report from a research work carried out in Southwestern Nigeria. The sandy loam soil is inherited from the parent material of the study area. The textural composition of soil is highly influenced by the parent material (Oguike and Mbagwu, 2009). According to Kiflu and Beyene (2003), soil textural composition is an inherited property of the soil that is not influenced over a short period of time. Sandy loam soils are made up of sand particles with varying amount of silt and clay (Table 1). It is the type of soil material that is good for planting rice because it normally allows for good drainage but cannot hold sufficient water and nutrients for healthy plant growth. It is also often deficient in some micro nutrients but with frequent irrigation and fertilizer, its beneficial properties are enhanced for agricultural purpose (ICSSD, 2019). In this study, nitrogen and organic matter content of the soil were low across the soil samples. This prompted the application of additional nutrients at different ratios, prior to microcosms and incubations. Organic matter and nitrogen content of any soil are important parameters used in judging soil quality and productivity. It has been reported to have significant positive influence on soil pH, colour, buffering capacity, base saturation, water holding capacity (Akamigbo, 1999) and effective cation exchange capacity (Onasanya, 1992).
Available phosphorous content of the soil was highest in Ebonyi South, followed by Ebonyi North and least on Ebonyi Central soil sample (Table 1). However, across the studied soil samples, the available P concentration in the soil was high on average, exceeding the critical level of phosphorous in soils of Southeastern Nigeria which is 15mg/kg (Enwezor *et al.,* 1990). The high available P concentration could be attributed to the continuous application of P-based fertilizer. This agrees with the findings of Tellen and Yerima (2018) who reported the highest value of available P in farming land-use. The high concentration of P across the soil samples under study especially in Ebonyi South and Ebonyi North is an indication that it may require little or no addition of phosphate fertilizer for increased rice yield.

The soil pore water as analyzed across the soil samples from the three zones of Ebonyi State revealed that lactate, formate, acetate, chloride, and propionate concentrations were higher in the planted pots, though these differences were only statistically significant for chloride and propionate. The significant level of chloride ion present in the pore water from our study corroborates the findings of Fixen (1993) who reported the presence of a significant concentration of chloride salt in rice paddy soil and suggested its enhancement of root biomass by 58% than sulfate salt, and its promotion of root growth. Furthermore, higher chloride salt than sulfate salt in paddy soil pore water exerts more osmotic pressure, but both has been reported to collectively have a positive correlation between osmotic pressure and root length. Also, higher osmotic pressure induced by chloride ion is suspected to be involved in the root growth promotion of rice and enhances more branching root morphology (Hassan and Overstreet, 1952; Cramer *et al*., 1986).

The concentrations of malate, nitrate, and sulfate were similar between rhizosphere (planted pots) and bulk soil (unplanted pots). In soil pore water the immediate source of nutrient uptake by plants and soil biota, oxygen released from rice roots serves as an electron acceptor for aerobic microorganisms and is likely to influence the microbial populations and the chemical changes that occur. The volume and composition of organic molecules released from roots changes in a way, the rice plant growth stage (Aulakh *et al.,* 2001), this theoretically could also cause

temporal shifts in the rhizosphere microbial community.

There is need to for an understanding of the native bacterial population, their characteristics, the distribution, and diversity of indigenous bacteria in the rhizosphere of rice plant in Ebonyi State. This is because microorganisms present in the rhizosphere play important role in the ecological fitness of the plant host, such important microbial processes that occur in the rhizosphere include plant protection/growth promotion as well as production of antibiotics, geochemical cycling of minerals and plant colonization. Exploring and unraveling the role of these microorganisms has opened a fascinating area of research in rhizosphere research.

The result of the morphological and biochemical identification of the isolates revealed different groups of microorganisms such as the gram positive and gram-negative group of bacteria within the study area. The gram-positive organisms were more abundant than the gram negatives. Data revealed that *Bacillus* spp and *Aeromonas* spp were the most suspected microorganisms across all the microbes observed in the rhizosphere. Similar findings on the abundance of *Bacillus* species in rice field soil samples have been reported by Seiphepo *et al.* (2013). This suggests that *Bacillus* spp are vital in nitrogen fixation in rice roots and soil which promotes rice growth. 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 that 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 correlates 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 (Seiphepo e*t al.,* 2013). This is because *Bacillus* can sporulate and so it can be formulated as a dry powder.

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 5). Phylogenetic analysis of the results revealed that some of the isolates belong to the phylum of Proteobacteria, a major phylum of Gram-negative bacteria. Madigan and Martinko (2005) reported Proteobacteria to include 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.

Conclusion

Since rice is one of the staple foods for more than half of the world population with growing demands all over the globe especially in developing countries, there is, therefore, the need to intensify rice cultivation to increase rice production to meet the global need for food security. Also, soil microorganisms have long been recognized as major players in nutrient recycling, absorption, and utilization in plant, hence, the study evaluated the effect of indigenous microorganisms on growth, physiology, and rice yield across the different geopolitical zones in Ebonyi State. This study showed the presence of sand as a major component of the soil samples from these zones studied and revealed mild acidic pH less than 6.0 but greater than 5.0. The study also revealed pore water content of many organic acids and inorganic acidic ions in both planted and unplanted soil from the three zones but at different concentrations. Isolation, identification, and

characterization of microbial community in the flooded rice soil sample was examined in this study. Bacillus species was suspected across the three zones. Phylogenetic analysis results revealed that some isolates are of Proteobacteria phylum, a major phylum of the Gram – negative bacteria and firmicutes which is a Gram positive bacterium.

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Contributions of authors:

UJN collected and processed the samples as well as carried out the literature search, OSC, UEN conceived, designed the study, wrote, and reviewed the manuscript. All authors read and approved the final manuscript.

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