Original Research Article

Nitrogen-fixing and phosphate solubilizing potentials of rhizobacteria from the rhizosphere of two cassava varieties in Iyamho community

Abstract

Microorganisms in close association with the roots of plants can enhance plant growth, through nitrogen fixation (NF) and phosphorus solubilization (PS). Although the type of microbes in close association with different plants vary which is a function of their growth and development. Therefore, in this study, the plant growth promoting properties of rhizobacteria present in the rhizosphere of two cassava varieties (Sweet cassava US, bitter cassava ST) indigenous to Iyamho community was explored. The samples were analysed for total heterotrophic bacteria community and the obtained isolates screened for NF and PS abilities using a semi-solid N-free medium and Pikovaskya agar respectively. The bacterial population in both agar medium varied, however, the bacterial counts on Luria Bertani (3.67×10^5, 3.35×10^6) was higher than in Nutrient agar (2.73×10^5, 2.68×10^5) after incubation for 24 hours at 37^oC for sweet and bitter cassava rhizosphere respectively. Also, isolates from sweet cassava had the highest bacteria count in both Nutrient agar and Luria Bertani agar. A total of sixteeen isolates were obtained, six phosphate solubilizers, five nitrogen fixers and five without traits for either NF or PS. The Gram-negative bacterial group was more dominant across all isolates while the dominant genus was *Bacillus* species. This study indicates that the nitrogen fixers and phosphate solubilizers are major constituents of the rhizomicrobe of cassava plants although the distribution varies across cassava variety. Sweet cassava rhizosphere harbored more nitrogen-fixing bacteria while both varieties had the same amount of phosphate solubilizing rhizobacteria.

Keywords: PGPR, rhizobacteria, Nitrogen-fixation, Phosphate solubilization, Cassava

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a major staple crop that has been designated as a 21st-century crop for smallholder farmers. It is one of over 100 trees, shrubs, and herb species of the genus *Manihot* estimated to have been imported from Argentina to the United States of America, according to the Food and Agriculture Organization of the United Nations (FAO) [1]. According to some other studies, cassava originated from the southernmost reaches of the Brazilian Amazon. Cassava is the most commonly cultivated tuber crop in the tropical region and a crop that consistently contributes to food security, owing to its ability to store matured edible roots in the ground for up to three years. It is unquestionably the world's sixth most important crop (after wheat, rice, maize, potato, and barley).

There are numerous cassava cultivars, which can be differentiated based on a variety of structural characteristics of the plant such as tuber shape, maturity date, yield, and the amount of cyanogenic glycoside present. *Manihot Phol* (bitter cassava) and *Manihot utilissima* (sweet cassava) are the two edible species with low and high hydrogen cyanide contents, respectively. According to McKey et al. [2], sweet cassava has less than 100 mg cyanogenic compounds per kilogram of fresh root, whereas 'bitter' cassava has more than 100 mg cyanogenic compounds per kilogram of fresh root. This hydrogen cyanide content can consequently affect the abundance and distribution of rhizobacteria in the cassava rhizosphere.

Cassava output in the world has recently risen to 291 million tonnes, with prominent countries such as Nigeria ranking first with 59 million tonnes in 2017 [3]. The current worldwide cassava productivity level of around 12 tonnes per hectare represents only 12% of its maximum production. Lack of scientific nutrient management and uneven crop nutrition are two major causes of the significant yield disparity. Various researches have been conducted to determine and fill the gap in cassava productivity, nutrient requirements, soil requirements, the evolution of fertilizer recommendations and their impact on bridging the yield gap. Nitrogen (N) and phosphorus (P) are two of the leading essential nutrients required for plant growth and development [4] However, only about 0.1 percent of total phosphorus in the soil is readily accessible for plant absorption despite the large quantity of inorganic and organic phosphorus in the soil [5]. Insoluble P can be converted to plant-available soluble P by phosphorus solubilizing bacteria.

Due to the limitation of these nutrients in the soil, fertilizers have been applied to increase crop yield over the years. These fertilizers have however caused major damage to the ecosystem due to several factors which generally contributes to environmental degradation, climate change and public health challenges, since less than 30% of chemical fertilizers applied are utilized by the plants, and the remaining 70% are either leached into the soil, run off into water bodies or emitted into the air [6, 7]. Various sustainable and non-invasive methods of adequately delivering nitrogen and phosphorus to effectively enhance plant growth are being sought by researchers, one of the sustainable methods globally adopted is the use of microbial inoculants indigenous to various plants which have the ability to either fix nitrogen, solubilize phosphate, or both, enhancing nutrient accessibility and uptake for plant utilization and growth development.

Bacteria, fungi, actinomycetes, protozoa, and algae are among the tiny life forms found in rhizosphere of plants. Bacteria are by far the most prevalent of these microbes (i.e., 95 percent). Bacteria associated with plants can affect plants in one of three ways, depending on the number of bacteria present: beneficial, detrimental, or neutral [8]. The plant rhizosphere is a small dynamic zone of soil that covers the plant root. It is characterized by a high abundance and diversity of microorganisms due to the availability of nutrients released by the plant roots which act as attractants for the diverse microorganisms [9]. These microbes which are referred to as plant growth promoting microbes help to modulate the chemical and physical components of the plants via several mechanisms such as nutrient uptake, hormone production, and antagonism against plant pathogens. Rhizomicrobes can help plants grow by supplying fixed nitrogen or phosphorus which are typically present in limited quantities in many soils. Microbial inoculants as biofertilizers are currently being studied as a suitable alternative to the synthetic based fertilizers for the enhancement of plant growth through biocontrol of pathogenic organisms, nutrient uptake and phytohormone production [10].

The rhizosphere of cassava contain a diverse range of bacteria including gram-negative bacteria, gram-positive Actinobacteria, and gram-positive non-Actinobacteria [11]. Thousands of different microbial communities cohabit in the rhizosphere of cassava roots, such as commensals, pathogens, and mutualists. Several rhizobacteria have been isolated from cassava rhizosphere as well as the rhizospheres of other plants and some of the most commonly reported rhizobacteria genera include *Bacillus, Azotobacter, Penicillum, Athrobacter and Azosporillim* [9]. Melo and Fiore [12] successfully cultivated 27 endophytic bacteria from cassava root using a culture-dependent technique and *Bacillus* predominated in the cassava root region according to this investigation. A similar technique was utilized in another related study by Leite and Pereira, [13] which discovered 28 bacterial assocaiated with the root of the plant. *Bacillus, Burkholderia, Enterobacter*, and *Pantoea* were the most common bacteria discovered in the cassava root. They all displayed biological features, such as the ability to solubilize inorganic phosphate.

Phosphate solubilizing bacteria and nitrogen-fixing bacteria have reportedly been used as biofertilizers and have been observed to increase the rate of Biological nitrogen fixation (BNF) in soils. Pueschel *et al.* [15], demonstrated that an increase in phosphorus acquisition in the legume–rhizobial symbiosis influenced the ability of arbuscular mycorrhiza fungi to increase the BNF activity of symbiotic diazotrophic bacteria. Phosphate solubilizing and nitrogen fixing bacteria can be efficiently used as biofertilizers due to their ability to aggressively colonize and establish on plant roots. Also, the richness and abundance of these beneficial rhizomicrobes is a function of the chemical composition of the rhizospheric region of plant which consequently affects the plant growth and yield. This study therefore comparatively assess the prevalence of nitrogen fixing and phosphate solubilizing bacteria in two variety of cassava cultivars.

MATERIALS AND METHODS

Study area

The experiment was carried out in Uzairue kingdom, specifically in Iyamho community which is a small town located in Etsako West Local Government Area of Edo State, Nigeria. The area is defined by latitude 70 20’ north of the equator and longitude 60 10’ East of the Greenwich meridian. Iyamho is a rural-urban community majorly constituted of farmers. The major crop grown in this coomunity amongst other plants is cassava.

Sample collection

Rhizospheric soil samples were collected from the rhizosphere of two varieties of cassava in Iyamho at 0-30cm depth with the aid of a calibrated soil auger and transferred into labeled sterile containers. The sample was transported to the laboratory at 4^0C within 2hrs of collection for microbiological analysis. The cassava stems and leaves were also taken to the plant biology and biotechnology Herbarium at Edo State University Uzairue for identification and characterization.

Enumeration of total culturable heterotrophic microorganisms

Enumeration of total culturable heterotrophic bacteria was conducted using the standard spread plate method. The soil samples after serial dilution were suspended in Luria Bertani (LB) medium and Nutrient agar (NA). Inoculated plates were incubated at 37^0C for 24 hours and colonies differing in morphological characteristics were enumerated and selected for further analysis [16].

Plant growth promoting activity

The phosphate solubilization index was calculated using the method of Pikovskaya [17]. Pikovaskaya agar was introduced as a thin film into a sterile petri dish and plates were inoculated with test isolates [18]. The formation of halo zones indicated a positive result for phosphate solubilization. The nitrogen-fixing ability of the isolates was determined according to the method of Ghevariya and Desai, [19]. All isolates were inoculated into a semisolid nitrogen-free medium. The bacterial strains were incubated for five days at 30^{o}C, pellicle growth was considered to be positive for N-fixation.

Identification and characterization of isolates

Bacterial colony morphology

Over the streak, typical bacterial colonies were observed. A single well-isolated colony was picked up and re-streaked onto freshly prepared Nutrient agar plates, and incubated in the same way for morphological assessment of each bacterial colony . The size, color, form, surface, elevation, and margin of colonies were determined.

Gram staining

All pure bacterial isolates were Gram-stained to characterize them into gram-positive or gram-negative bacteria. Gram staining displays bacterial morphology (cocci, rods, or spiral-shaped bacteria) and differentiates gram-positive (violet-stained) and gram-negative (red-stained) bacteria based on cell wall structure (peptidoglycan layer thickness variations) and permeability [16].

Biochemical tests

Biochemical tests were conducted to further identify the bacterial isolates; the following biochemical tests were conducted according to the method of Ju *et al.* [20]. Catalase, Oxidase, Urease test, motility test, Nitrate reduction test, citrate test, Indole test, Methyl red, and sugar tests (Glucose, Maltose, Mannitol, sucrose, lactose).

RESULTS

Total culturable bacterial Isolates

Total culturable bacterial isolates were enumerated on nutrient agar and Luria Bertani agar medium. The isolates were labeled as US and ST for the Sweet and Bitter cassava rhizosphere isolates respectively. The bacteria colonies isolated from the two varieties of cassava on Luria Bertani recorded more growth than that of nutrient agar. The colony-forming units (CFU) are indicated in Table 1.

Table 1 Total culturable bacterial isolates from the rhizosphere of two cassava cultivars using Luria Bertani and Nutrient agar

Cassava rhizosphere	Nutrient agar	Luria Bertani agar
ST	**2.73×10^5**	**3.67×10^5**
US	**2.78×10^5**	**3.35×10^6**

Plant growth promoting activity

Since rhizobacteria can provide plants with other essential macro-and micronutrients, all of the strains isolated in this study were tested for their ability to solubilize phosphate (PO_4) and fix nitrogen in a semisolid N-free medium. Isolates ST3, ST4, US1, US5, US6, and US9 had the ability to solubilize the phosphorus while isolates ST2, ST6, US3, US4, and US8 all showed positive results for nitrogen fixation. The plant growth promoting properties of the test bacterial isolates are presented in Figure 1. The percentage of the nitrogen fixing and phosphate solubilizing rhizobacteria is clearly described in Table 2.

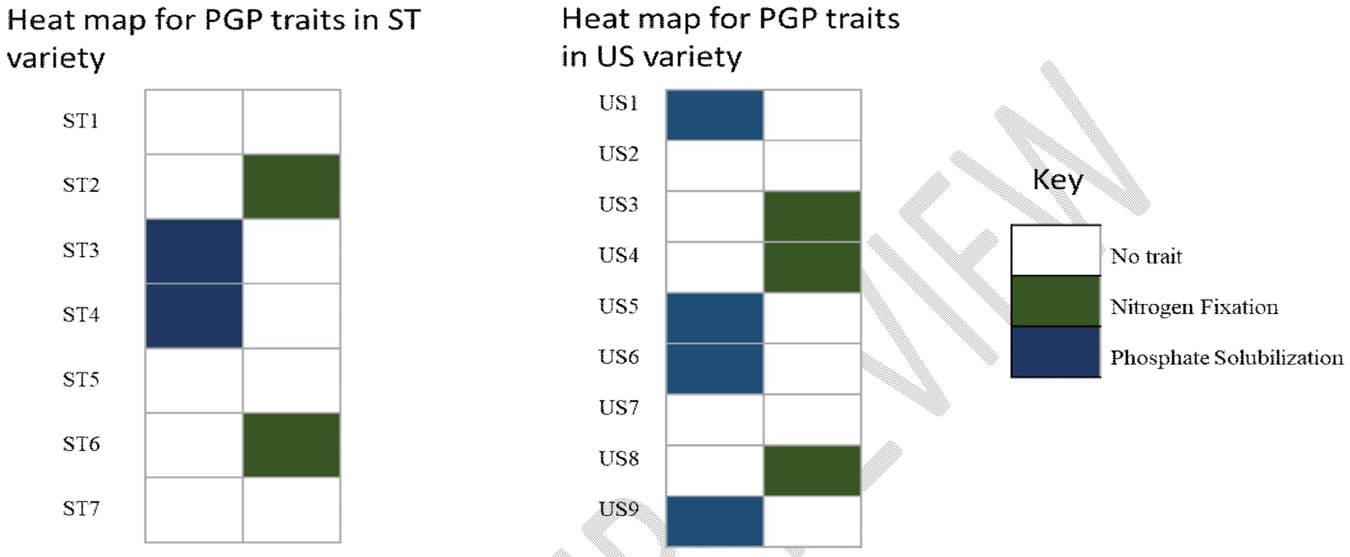

Figure 1. Heat map for the N-fixing and P-solubilizing abilities of rhizospheric bacteria in both cassava varieties

Table 2 Percentage of Nitrogen-fixing and Phosphate solubilizing rhizobacteria

Cultivar	**Total Number of bacterial isolates with NF and PS traits**	**N.F**	Percentage (%)	P.S	Percentage (%)	**No trait**	**Percentage (%)**	**Total Number of rhizobacteria**
ST	**4**	2	28.57	2	28.57	3	42.86	7
US	**7**	4	44.45	3	33.33	2	22.22	9
Total	**11**	6	73.02	5	61.90	5	65.08	16

Key: N.F/Nitrogen fixation, P.S/ phosphate solubilization

Identification and characterization

Sixteen (16) bacteria were isolated from the soil samples ou of which eleven (11) showed traits for either N.F. or P.S. The eleven isolates were further identified using biochemical and colonial morphological properties. The bacteria showed varying colony sizes from tiny, small to large, with colors ranging from white, pale yellow to pink. Some colonies were grayish, mucoid, and flat**.** Colonial morphological properties of N.F. and P. S. bacterial isolates are presented in Table 3 while the result of the biochemical properties and tentative identification of the nitrogen fixing and phosphate solubilizing rhizobacterial isolates are presented in Table 4.

Table 3 Colonial morphological properties of N.F. and P. S. bacterial Isolates

Rhizobacterial isolates	Shape	Color	Elevation	Surface	Margin	Transparency	Consistency	Diameter
ST2	Round	Yellow	Flat	Smooth	Entire	Opaque	Dry	Small
ST3	Round	Cream	Raised	Smooth	Entire	Opaque	Dry	Large
ST4	Round	White	Raised	Smooth	Entire	Opaque	Dry	Medium
ST6	Round	Cream	Raised	Smooth	Entire	Clear	Dry	Small
US1	Round	Cream	Flat	Smooth	Wavy	Opaque	Dry	Medium
US3	Round	Pink	Flat	Smooth	Entire	Opaque	Dry	Medium
US4	Round	White	Flat	Smooth	Entire	Clear	Slimy	Large
US5	Round	Cream	Flat	Rough	Wavy	Opaque	Dry	Small
US6	Round	White	Flat	Smooth	Entire	Opaque	Dry	Medium
US8	Round	Pink	Flat	Smooth	Entire	Clear	Slimy	Medium
US9	Round	Cream	Raised	Smooth	Entire	Opaque	Dry	Small

Table 4 Biochemical identification of Bacterial Isolates

Isolates	Gram stain	Shape	Oxidase	Catalase	H_2S	Citrate	Urease	Indole	Glucose	Sucrose	Lactose	Maltose	Fructose	Tentative Identification
ST2	-	r	-	+	+	-	-	+	+	-	+	+	+	*Bacillus* spp.
ST3	+	r	-	+	+	-	-	-	+	+	+	+	+	*Bacillus* spp.
ST4	+	r	-	+	-	-	+	-	+	+	+	+	+	*Bacillus* spp.
ST6	-	r	-	+	-	+	+	-	+	+	-	+	+	*Pseudomonas* spp.
US1	+	r	-	+	-	-	+	-	+	+	+	+	+	*Bacillus* spp.
US3	-	r	-	+	-	+	+	-	+	+	-	+	+	*Serratia* spp.
US4	-	r	+	+		+	-	-	+	-	-	+	-	*Pseudomonas* spp.
US5	-	c	+	-	+	+	-	-	+	-	+	+	-	*Agrobacterium* spp.
US6	-	r	+	+	-	-	-		-	-	+	-	-	*Escherichia* spp.
US8	-	r	-	+	-	+	+	-	+	+	-	+	+	*Serratia* spp.
US9	-	r	-	+	+	-	-	-	-	+	+	+	+	*Bacillus* spp.

Key = r: rod, c: cocci, +: positive, -: negative

DISCUSSION

Plant growth promoting microbes increase plant growth and reduce susceptibility to diseases by colonizing the rhizosphere of plants [21]. These microorganisms are able to promote plant growth and development through several mechanisms. These mechanisms could be direct mechanisms such as nitrogen fixation, siderophore production, phosphate solubilization, phytohormone production, or other indirect mechanisms such as antibiotics production, induced systemic resistance, lytic enzyme degradation, exopolysaccharides production. The total culturable heterotrophic population determined on N.A and L.B agar media revealed that a higher population of bacteria counts was observed in sweet cassava variety than the bitter cassava variety for both media used. Reports have shown that sweet cassava roots contain less than 50 mg per kg hydrogen cyanide (HCN) on fresh weight basis, whereas that of bitter variety may contain up to 400 mg per kg HCN. Hydrocyanic or prussic acid (HCN) is a toxic metabolite produced in cassava, when two major cyanogenic glucosides (linamarin and lotaustralin) present in the cassava root is hydrolysed and released into the rhizosphere. This root-secreted metabolite has the potential to shape the root microbiome and modulate the microbial community population due to its high level of toxicity [32]. The toxic nature of the HCN has potentials of shaping the microbial community within the plant which could be the reason for lower microbial population reported in the bitter cassava rhizosphere. Luria-Bertani broth is a common rich medium among bacteriologists since it allows for rapid growth and high yields of a variety of species. Also, the microbial growth in L.B. was higher for both sweet and bitter cassava than N.A. Although N. A. is a general purpose medium that supports the growth of a large array of microorganisms, L.B. is the most suitable for isolating bacterial from rhizospheric soil and its chemical composition include tryptone, yeast extract, and sodium chloride (NaCl) which has sufficient levels of all important inorganic nutrients required for the growth of rhizomicrobes [31].

A total of 16 bacteria were isolated from the rhizosphere of both cassava cultivars, out of which 11 showed positive trait for either N. F. or P. S, 7 from sweet cassava (4 N.F. and 3 P.S. bacteria) and 4 from bitter cassava (2 each for N. F. and P. S.). From the result, above 50% of the total population of rhizobacteria from both cassava cultivars harbored either of the traits. This shows that the cassava rhizosphere is a rich ecological niche for PGPR. The higher rhizobacterial population, diversity and plant growth promoting activity captured by isolates from the sweet cassava rhizosphere as compared to the bitter cassava have been suggested to be due to the high hydrogen cyanide contents in the bitter cassava which may inhibit the growth of root-associated plant growth promoting bacteria and also suppress nitrogen fixation and phosphate solubilizing attributes which are highly beneficial to plant growth and development [11].

Inoculation of plants with nitrogen-fixing bacteria has caused a significant increase in nutrient status and plant biomass in bamboo, potato, and maize through biological nitrogen fixation [22]. According to Kumar *et al.* [16], co-inoculation of wheat with nitrogen-fixing and p-solubilizing rhizobacteria increased wheat growth in both greenhouse and field conditions since phosphate and nitrogen gas are majorly in forms not naturally accessible to plants. Phosphate solubility is a common trait through which rhizobacteria could substantially enhance plant growth. In an experiment by Gupta *et al.* [23], isolates from the rhizospheric soils of cassava (*Azotobacter*) and maize (*Serratia*) harbored diverse plant growth promoting traits including phosphate solubilization. This justifies that these isolates secretes organic acids and phosphatases responsible for solubilizing the insoluble phosphate to soluble forms. Consequently, phosphorus is an essential nutrient for plant growth and can be made available for roots to sustain plant growth. This result correlates with findings of Chinakwe *et al.* [24] and previous work by Ehis-Eriakha *et al.* [18].

The nitrogen-fixing and p-solubilizing ability of the bacteria from the rhizosphere of the two different cassava cultivars varied. According to Safriani *et al.* [26], soil types and plant species are major factors affecting microbial community distribution and function in the rhizosphere. Thus, the cassava variety may influence the diversity and population of rhizobacteria resident in the rhizosphere due to the composition of the root exudates released by the plant..

Also in this study, isolates with nitrogen fixing and phosphate solubilizing potentials from both cassava varieties were identified using conventional colonial morphological and biochemicla techniques. Some of the organisms identified belonged to the following genera: *Bacillus*, *Agrobacterium, Pseudomonas, Serratia* sp with *Bacillus* being the most dominant species. From the bitter cassava rhizosphere, *Bacillus* and *Pseudomonas* were the identified genera while, *Bacillus, Serratia, Agrobacterium* and *Pseudomonas* were obtained from sweet cassava. *Bacillus* and *Pseudomonas* species showed the ability fix nitrogen and solubilize phosphate which is in tandem with the research conducted by Sibponkrung, *et al.* [27] to evaluate the nitrogen-fixing ability of *Bacillus velezensis* S141 co-inoculated with *Bradyrhizobium diazoefficiens* USDA110 on soybean. They observed an increase in nodule formation and nitrogen fixation which led to the formation of larger nodules and increased yield. Also, according to Miljaković, *et al*, [28], *Bacillus* sp was reported to promote plant growth via nitrogen fixation enhancement. Majority of the phosphate solubilizing rhizobacterial isolates from both sweet and bitter cassava varieties (66.67%) were identified as *Bacillus* species. Similarly, *Pseudomonas* has been isolated, characterised and applied as a known PGPR. In a study by Moustaine *et al.* [29], a single strain of *Pseudomonas agglomerans* solubilized phosphate in vitro in Petri plates by creating a distinct halo around the colony. The application and potential of rhizospheric bacteria have largely been acknowledged and authenticated across the globe in the last few decades as non-invasive eco-friendly and sustainable alternative to synthetic base fertilizers.. Stimulation of plant growth and yield by rhizospheric bacteria has been reported at laboratory, greenhouse, and field levels in several studies [30, 31].

CONCLUSION

The results of the research showed that about 60% of the organisms isolated from the two cassava rhizosphere were able to either fix nitrogen or solubilize phosphate. The study reveals more information on the availability and abundance of plant growth promoting rhizobacteria in the rhizosphere of different cassava varieties in Iyamho community. The rhizosphere of both the sweet and bitter cassava varieties are replete with rhizobacteria with nitrogen fixation and Phosphate solubilization abilities. However, the sweet cassava rhizosphere harbored more Nitrogen fixers and Phosphate solubilizers. This research is important because it suggests that indegineuos rhizomicrobes which are capable of enhancing the growth of plants are resident in the rhizosphere of plants and may vary in their diversity, abundance, and functions depending on the plant varieties. These rhizobacteria can however be harnessed as a sustainable alternative growth enhancement option such as biofertilizers or as an inoculant to replace the use of chemical fertilizers to boost productivity in local agro-climatic conditions.

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