Original Research Article

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

Abstract

Microorganisms in close association with the roots of plants have the ability to enhance plant growth, through nitrogen fixation (NF) and phosphorus solubilization (PS). However, there is a paucity of information on the role and potential of bacterial communities indigenous to the rhizosphere of cassava plants in Iyamho community. This study presents the assessment of the rhizobacteria of two cassava cultivars for their NF and PS abilities. The rhizospheric bacteria were isolated from the soil-root region of two cassava varieties identified as sweet and bitter cassava. The bacteria species were isolated on Nutrient agar and Luria Bertani agar for effective isolation of all possible cultural species. All bacteria isolated were screened for nitrogen-fixation and phosphate solubilization ability 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 in both isolates from the sweet and bitter cassava rhizosphere respectively. Isolates from sweet Cassava had the most bacteria count in both Nutrient agar and Luria Bertani agar. Out of the sixteen bacteria isolated, six were phosphate solubilizers while five were nitrogen fixers and the Gram-negative group of bacteria was more predominant. Amongst all isolates, 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 in Iyamho, thus can be used to enhance plant growth and promote crop yield. Sweet cassava rhizosphere also harbored more Nitrogen-fixing bacteria while both varieties had the same amount of Phosphate solubilizing rhizobacteria.

Keywords: PGPR, Diazotrophs, 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, 2013). According to some other studies, cassava originates 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. Other characteristics used to characterize the many kinds of the plant include tuber shape, maturity date, yield, and the amount of cyanogenic glycoside present. *Manihot Phol??? (Check it it may be variety?)* and *Manihot utilissima* or sweet cassava are two edible species with low and high hydrogen cyanide contents, respectively. 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 (Otekunrin and Sawicka, 2019). 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, and the evolution of fertilizer recommendations and their impact on bridging the yield gap. Nitrogen (N) and phosphorus (P) are two of the major essential nutrients required for plant growth and development (de Almeida *et al.*, 2021). However, only about 0.1 percent of total Phosphorus in the soil is readily accessible for plant absorption although there may be a large quantity of inorganic and organic phosphorus in the soil (Li, *et al.*, 2017). 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 the excess quantity applied since less than 30% of chemical fertilizers applied are utilized by the plants (Sharma *et al.*, 2013) and the remaining 70% are either leached into the soil or run off into water bodies. The negative effects may include the loss of soil fertility and eutrophication which occurs when the excess fertilizers are washed from the soil surface into water bodies (Schindler *et al.*, 2008). 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 which are globally being adopted is the use of microorganisms indigenous to various plants which have the ability to either fix nitrogen, solubilize phosphate, or both, making these nutrients accessible for plant utilization and growth development.

Bacteria, fungi, actinomycetes, protozoa, and algae are among the tiny life forms found in the rhizosphere of plants. Bacteria are by far the most prevalent of these microbes (i.e., 95 percent). Soil contains a significant number of bacteria (about 10^8 to 10^9 cells per gram of soil), yet the amount of cultural bacterial cells is only around 1% of the total number of cells present. The bacteria in a soil sample can affect plants in one of three ways, depending on the number of bacteria present: beneficial, detrimental, or neutral (Mehmood *et al.*, 2018). The plant rhizosphere is a tiny 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 (Belay and Teshome, 2021). These 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 and are referred to as plant growth promoting rhizomicrobes. Rhizospheric microorganisms such as plant growth promoting rhizobacteria (PGPR) and fungi can help plants grow by supplying fixed nitrogen or phosphorus, both of which are typically present in small levels in many soils and are currently being studied as a suitable alternative to the synthetic based fertilizers for the enhancement of plant growth and biocontrol of pathogenic organisms (Khan *et al.*, 2018). 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. According to Pueschel *et al.* (2017) by increasing phosphorus acquisition in the legume–rhizobial symbiosis, arbuscular mycorrhiza has the ability 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.

Several plant rhizobacteria have been isolated from diverse rhizospheres and characterized and some of the most commonly reported rhizobacteria genera include *Bacillus, Azotobacter, Athrobacter, Azosporillim* (Belay and Teshome, 2021). In previous research by Wang *et al*. (2013), a diazotrophic bacteria *Paenibacillus beijingensis* BJ-18 was isolated from the wheat rhizosphere which had high nitrogenase activity. According to Xie *et al.* (2016), the isolate also showed various biocontrol activities against plant pathogens. Li *et al.* (2017) in an experiment also isolated PGPR from the Maize rhizosphere which had the ability to solubilize organic and inorganic phosphorus and could further promote plant growth in Tomato and maize.

Materials and methods

Study area

The experiment was carried out in the Uzairue kingdom, specifically in Iyamho. Iyamho is a small town located in the 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. Cassava, which is a major staple crop in Nigeria, is the major crop produced by farmers in Iyamho.

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 ^{o}C 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 Uzaiure for Identification and characterization.

Enumeration of total cultural heterotrophic microorganisms

Enumeration of total cultural heterotrophic bacteria was conducted using the standard spread plate method. The soil samples after serial dilution were suspended in Luria Bertani (LB) medium. Inoculated plates were incubated at 37 ^{o}C for 24 hours and colonies differing in morphological characteristics were enumerated and selected for further analysis (Kumar *et al.*, 2015).

Plant growth promoting activity

The phosphate solubilization index was calculated using the method of Pikovskaya (1948). Pikovaskaya agar was introduced as a thin film into a sterile petri dish and plates were inoculated with test isolates (Ehis-Eriakha *et al.,* 2020). 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, (2014). 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

Over the streak, typical bacterial colonies were observed. A single well-isolated colony was picked up and re-streaked onto fresh LB and Nutrient agar plates, and incubated in the same way. On nutrient agar plates, the morphological characterization of each bacterial isolate was investigated. The size, color, form, surface, elevation, and margin of colonies were determined using a three-day-old bacterial isolate culture. After 24 hours, the color and shape of the colonies were recorded.

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 (Ehis-Eriakha *et al.*, 2020)

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.* (2020). Catalase, Oxidase, Urease test, motility test, Nitrate reduction test, citrate test, Indole test, Methyl red, and sugar tests (Glucose, Maltose, Mannitol, sucrose, lactose)

Results

A total of sixteen Bacterial colonies were isolated from the rhizosphere of two varieties of cassava using 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 counts are presented in Table 1.

Table 2 represents the bacteria species that were identified from the soil samples. Meanwhile, sixteen bacteria species were isolated from the soil samples and were labelled as ST1 – ST7, and US1 - US9. But after characterization with cultural, morphological and physiological tests, seven prominent bacteria species were identified where ST2, 3 and 4 was *Bacillus subtilis*, ST 6 and US4 was *Pseudomonas alcaligenes*, US1 was *Bacillus cereus*, US3 and US8 was *Serratia marcescens*, US5 was *Agrobacterium tumefaciens*, US6 was *Escherichia coli*, and US9 as *Bacillus megaterium*.

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). Isolates ST3, ST4, US1, US5, US6, and US9 had the ability to solubilize the phosphorus. All of the strains isolated in this study were tested for their ability to fix Nitrogen in a semisolid N-free medium. 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 (Table 3).

Figure 1 represent the heat map for the N-fixing and P-solubilizing abilities of rhizospheric bacteria in two cassava varieties. Isolates ST2, ST6, US3, US4, and US8 were found as nitrogen fixing bacteria species, ST3. ST4, US1, US5, US6 and US9 were found as phosphate solubilizers, while isolates STI, ST5, ST7, US2 and US7 were of no trait.

Discussion

Some bacteria species enhance plant growth and reduce susceptibility to diseases by colonizing the rhizosphere of plants (Goswami *et al.,* 2016). These plant growth promoting rhizobacteria 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, exopolysaccharides production. In this study, all the bacteria isolates from the Cassava rhizosphere were screened using microbiological culture methods for their ability to fix Nitrogen fixation and solubilize phosphate. None of the isolates tested positive for both phosphate solubilization and nitrogen fixation. 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 as reported by Ke *et al*. (2019). According to Kumar *et al.* (2017), co-inoculation of wheat with nitrogen-fixing and p-solubilizing rhizobacteria increased wheat growth in both greenhouse and field conditions.

Phosphate is majorly in a form that is not accessible to plants. Phosphate solubility is a common trait through which rhizobacteria could substantially enhance plant growth. In an experiment by Gupta *et al.* (2015), isolates from the rhizospheric soils of cassava (*Azotobacter*) and Maize isolate (*Serratia*) harbored diverse plant growth promoting traits including phosphate solubilization. This justifies that these isolates secrete organic acids and phosphatases, which solubilize the insoluble phosphate. Consequently, phosphorus is an essential nutrient for plant growth and can be made available for roots to sustain plant growth. This result is in line with the results of the work conducted on Maize in Nigeria by Chinakwe *et al.* (2019) and previous work by Ehis-Eriakha *et al.* (2020)

The nitrogen-fixing and p-solubilizing ability of the bacteria from the rhizosphere of the two different varieties of the cassava varied. According to Eviati and Sulaeman, (2009)??? Authors marked place???Why??, P content available in cassava rhizosphere (4.20 mg kg^{-1}) soils is classified as a low category. Low levels of available P may be caused by low organic material present in the soil. Biotic and abiotic factors are assumed to affect the structural and functional diversity of bacterial communities. According to Safriani *et al.* (2020), soil types and plant species are major factors affecting microbial communities in the rhizosphere. Thus, the cassava variety may influence the diversity and population of rhizobacteria resident in the rhizosphere.

The isolated bacteria species in this study were able to fix nitrogen and this is in tandem with the research conducted by Sibponkrung, *et al.* (2020) to evaluate the nitrogen-fixing ability of *Bacillus velezensis* S141 co-inoculated with *Bradyrhizobium diazoefficiens* USDA110 on soybean. In their study, they observed an increase in nodule formation and Nitrogen fixation which led to the formation of larger nodules. Also, according to Miljaković, *et al*, (2020), *Bacillus* sp has been reported to promote plant growth via nitrogen fixation enhancement.

In a study by Goswami *et al.* (2016), a single strain of *P. agglomerans* could solubilize phosphate in vitro in Petri plates by creating a distinct halo around the colony. Selected strains also tested positive for Nitrogen fixation by changing the green color of the medium based on malic acid in a blue environment. The application and potential of rhizospheric bacteria have largely been acknowledged and authenticated across the globe in the last few decades.

Stimulation of plant growth and yield by rhizospheric bacteria has been reported at laboratory, greenhouse, and field levels in several studies (Figueiredo *et al*., 2016).

Conclusion

The results of the research showed that about 60% of the organisms isolated from the two cassava rhizospheres 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 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.

Table 1: Total cultural bacterial isolates using Luria Bertani and Nutrient agar (CFU/g)

Cassava rhizosphere	Nutrient agar	Luria Bertani agar
ST	2.73×10^5	3.67×10^5
US	2.68×10^5	3.35×10^6

Table 2: Biochemical identification of Bacterial Isolates

Isolates	Gram stain	Shape	Oxidase	Spore stain	Catalase	Motility	Nitrate reduction	H_2S	Starch hydrolysis	Citrate	Urease	Indole	Glucose	Sucrose	Lactose	Maltose	Galactose	Fructose	Tentative Identification
ST2	+	r	-	+	+	+	+	+	+	-	-	-	A	A	-	A	AG	AG	***Bacillus subtilis***
ST6	-	r	+	-	+	+		+	-	+	+	-	-	+	-	+	-	+	***Pseudomonas alcaligenes*.**
US1	+	r	-		+	+		-		-	+	-	A	-	-	A	A	AG	***Bacillus cereus***
US3	-	r	-	-	+	+	-	-	-	+	+	-	A	+	-	+	-	+	***Serratia marcescens***
US5	-	r	+	-	-	-	-	+	-	+	-	-	AG	-	A	A	A	A	***Agrobacterium tumefaciens***
US6	-	r	+	-	+	+	-	-	-	-	-	+	-	-	A	-	A	A	***Escherichia coli***
US9	-	r	-	+	+	+	+	+	+	-	-	-	A	A	-	A	A	AG	***Bacillus megaterium***

Table 3 Percentage of Nitrogen-fixing and Phosphate solubilizing rhizobacteria

Cultivar	Total Number of bacterial isolates	N.F	Percentage (%)	P.S	Percentage (%)
ST	7	4	57.14	2	28.57
US	9	3	33.33	2	22.22

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

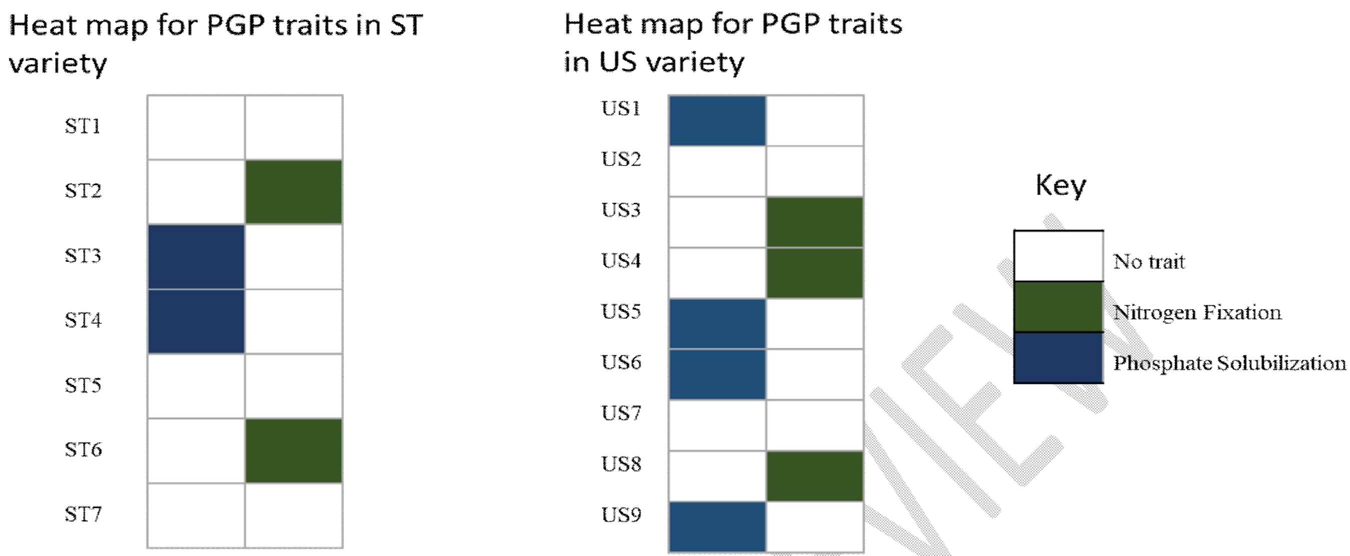

Fig 1. Heat map for the N-fixing and P-solubilizing abilities of rhizospheric bacteria in two cassava varieties

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