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ISOLATION AND CHARACTERIZATION OF RHIZOBIA STRAINS FROM THE ROOT NODULES OF GROUNDNUT (*Arachis hypogaea* L.) VARIETIES AT BAYERO UNIVERSTY RESEARCH FARM, KANO STATE, NIGERIA

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ABSTRACT
The study was carried out to isolate and identify Rhizobia phenotypically and biochemically from the root nodules of Groundnut (Arachis hypogaea L.) at Bayero University research farm. During the study rhizobia species were isolated from Groundnut varieties. Rhizobia strains were morphologically characterized based on their shape, size, borders, texture, nature of colony, margin, color, mucosity, transparency, and elevation. Biochemical characterization was conducted in which Gram staining, Congo red test, reaction of Yeast extract mannitol agar with bromothymol blue, cultured in peptone agar plate tests were conducted. Other biochemical characterization techniques carried out were growth pH, Temperature tolerance, growth in yeast extract agar were also carried out. The strains were molecularly characterized by DNA extraction, polymerase chain reaction (PCR) and Gel electrophoresis. The colony color ranges from white to yellow with entire margin, smooth colony surface and all the isolates produced mucous, rod to round shape and translucent to transparent. Thirty (30) isolates were Gram negative, produced white colonies when reacted with yeast extract agar. Most of the isolates absorb little Congo red hence produced pale to pale pink colors and few isolates absorbed Congo red strongly with deep black color indicating the presence of Agrobacterium as a contaminant. Isolate from Jar bahaushiya variety was 99.151% similar with Bradyrhizobium elkani, Mesorhizobium loti, Allorhizobium vitis and Sinorhizobium melitoti, Agrobacterium radiobacter and Agrobacterium vitis with a sequence length of 627bp. It can be inferred that to identify rhizobia strains which are suitable for bioinoculant production, characterization of rhizobia is a prerequisite. Hence, effective strains can be identified to boost agricultural productivity.
Key words: Rhizobium strain, Characterization, Morphological, Biochemical

INTRODUCTION

Groundnut *(Arachis hypogaea L.)* belongs to the family fabacaea (leguminosae). It is a multipurpose grain legume, which is considered a nutritious component in diets and a source of income for smallholder farmers in developing countries. It is either grown for its nut, oil, and its vegetable residue (haulms). It also provides high quality cooking oil and is an important source of protein for both human and animal diet and provide much needed foreign exchange by exporting kernels and cake (Nautiyal, 1999).

Rhizobia are bacteria that fix nitrogen (diazotrophs) after becoming established inside root nodules of legumes (Fabaceae). They are gram negative, motile and nonsporulating rods. It required a plant host; they cannot independently fix nitrogen. It forms endosymbiotic nitrogen fixing association with root of legumes.

Despite the importance of nitrogen fixation to the global nitrogen balance, very few Rhizobia strains have been completely sequenced and characterized representing less than 1% of the complete bacterial genomes available today. Very few genomics studies comparing nitrogen fixating bacteria have been performed (Guerrero *et al.*, 2005; Carvalho *et al.*, 2010). Positive response had been reported when legumes such as cowpea and soy bean were inoculated with rhizobia strains. Good results with groundnut have been obtained elsewhere in the world, there is little information in Sudan savannah of Northern Nigeria.

The symbiotic relationship between legumes and nitrogen fixing bacteria is critical for agriculture, as it may have profound impact on lowering cost

of farmers, land sustainability, soil quality and mitigate greenhouse gas emissions. Consequently, there has recently been a growing level of interest in environmentally friendly sustainable agricultural practices and organic farming systems (Rigby *et al.,* 2001; Lee *et al.,* 2007). Increasing and extending the role of biofertilizers such as *Rhizobium* would *Rhizobium* inoculants significantly improves yield in many leguminous crops and can minimize the use of synthetic fertilizer which is rather expensive and deteriorates soil properties (Laurette, 2015). Their ability to fix nitrogen in symbiosis makes them excellent colonizers of low nitrogen environment and economically friendly crop pasture (Jensen and Hauggaard, 2002). In addition, nitrogen from legume fixation is essentially "free" for use by both the host plant and associated or subsequent crops (Kiers *et al.,*2002). A well-established practice for maintaining soil fertility has been the cultivation of leguminous plants which replenish atmospheric nitrogen through symbiosis with Rhizobium species in rotation with non-leguminous plants (Deka and Azad 2006).

This study was carried out to isolate and characterized nitrogen-fixing bacterium *(Rhizobium* spp.) phenotypically and biochemically as characterization of rhizobia is a prerequisite to bioinoculant production to increase crop yield.

MATERIALS AND METHODS

Study Area

The research was conducted at BUK, research farm Kano State with latitude 11^0 96′33′18 N and longitude 8^0 41′40″16 E. Phenotypic and biochemical characterization was conducted at Microbiology laboratory of the Department.

Agronomic Practice

Land was ploughed with a hoe to establish fine tilth. In the field,4m length of the ridges with 0.75m between the ridges were made which was equivalent to 4m x0.75m =3m width. The width was 3m which was equivalent to $12m^2$ area per plot, each variety was replicated three times which gave a total of $12m^2x3=36$ m^2 for one variety of groundnut, for the ten varieties was equivalent to $360m^2$ Two seeds were sown in each hole. Weeding was conducted fifteen days after planting. Harvesting was manually done by pulling the groundnuts from the soil (Makate *et al.*, 2018).

decrease the need for chemical fertilizers and reduce adverse environmental effects. *Rhizobium* symbiosis with legumes species is of special importance, producing 50% of 175 million tons of total biological nitrogen fixation, annually providing nearly half of all N used in agriculture (Hatice *et al.,* 2008).

Sample Collection and Pretreatment

A clean spade was used to dig approximately 15 cm to either side of the plant stalk to a depth of at least 20 cm. The clump of soil and roots were lifted out carefully, placed in clean plastic bags and kept in an ice box. It was then transported to Bayero University Kano, Microbiology laboratory for isolation, morphological and biochemical characterization.

Preparation of media

Yeast extract agar of 5.75 g was measured and suspended in 250ml of distilled water and was heated to boiling to dissolve the medium. It was sterilized by autoclaving with prestige medical series 2100 clinical autoclave at 15bs pressure at a temperature of 121^{o}C for 15 minutes, it was cooled to 50 ^{o} C well mixed and poured in to sterile petri plates as prescribed by the manufacturer.

Isolation of Rhizobia Strains

It was isolated following the protocol of Vincent (1970) and Preyanga *et al.* (2019)

Morphological Characterization

The colony morphology of the isolates was examined as prescribed by Vincent, (1970) and Justine *et al.* (2020).

Biochemical Identification

Gram Staining Test

It was carried out following the method described by Harold (2002).

Congo Red Absorption

The ability of the isolates to absorbed Congo red chemical was carried out based on the protocol of Vincent, (1970) and Halima *et al. (*2012).

Reaction with Bromothymol Blue in Yeast Extract Mannitol Agar

It was determined as prescribed by Beck *et al.* (1993) and Preyanga *et al.* (2019). The plates were incubated in YEMA medium containing bromothymol blue (YEMA-BTB) at 28°C for 7 days in the dark. The color changed was observed and recorded.

Cultured in Peptone Glucose Agar Plates

Rhizobia isolates were cultured in peptone glucose agar plates, incubated at 28°C for 4 days in the dark. (Beck *et al*., 1993) and Gilbert *et al.* (2018).

Salt tolerance test

Rhizobia isolates were examined for their tolerance to salt on yeast extract mannitol agar (YEMA) supplemented with 0.5, 1, 1.5, and 2% (w/v) NaCl as described by Ben Romdhane *et al,* (2006).

pH tolerance test.

The ability of the *Rhizobia isolates* to grow on media at several pH values were tested by streaking cultures on the YMA plates, where the pH values were adjusted to 4.8, 5.8, 6.8, and 8.8 with either NaOH or HCl (Küçük *et al*., 2006).

Temperature tolerance

The inoculated plates of *Rhizobia* isolates were incubated at 4, 20, 27, 37 and 55°C as prescribed by Hung *et al.* (2005).

Reaction with yeast extract agar

The isolates were examined on yeast extract mannitol agar (YEA) plate, after it was incubated for a period of 3 to 7 days at 28°C. The color change was examined and recorded as described by Vincent (1970).

Starch Hydrolysis Test

It was conducted as prescribed by De oliveira, (2007).

Molecular Characterization

DNA Extraction

It was conducted according to a protocol of Doyle and Doyle, (1990) and Devendra *et al.* (2020).

Polymerase Chain Reaction (PCR)

It was carried out based on the method conducted by Doyle and Doyle, (1990) and Devendra *et al.* (2020).

Gel Electrophoresis of PCR

It was performed according to a methodology carried out by Doyle and Doyle, (1990) and Devendra *et al.* (2020).

RESULTS

Table 1 shows morphological properties of Rhizobia isolates from Groundnut varieties (*Arachis hypogaea L*). When viewed under microscope, it was observed that the shape of the isolates ranged from rod to round shape. Most of the isolates were translucent and some were transparent. The colony color ranges from white to yellow with entire margin, smooth colony surface and ten (10) isolates produced mucous.

Table 2 shows biochemical characterization of Rhizobia species isolated from root nodules of Groundnut varieties (*Arachis hypogaea* L.). The result revealed that, ten (10) isolates were gram negative produced white colonies when reacted with yeast extract agar. Most of the isolates did not absorb Congo red hence, produced pale to pink color. Few isolates absorb Congo red strongly produced deep black color. Few isolates produced blue color when yeast extract agar was added to bromothymol blue. Whereas most of the isolates produced yellow color. The isolates were able to tolerate salt concentration within the range of 0.5-1.9 % (w/v), with the optimum growth pH of 4.9-9.5. Most of the isolates showed no growth when cultured in peptone glucose agar plate and few showed growths. The isolates were able to with stand optimum temperature of 19-35^{o}C.Ten isolates produced clear zone when iodine solution was added (hydrolysis test)

Table 3 shows the result of molecular characterization of Rhizobia strains isolated from root nodules of Groundnut (*Arachis hypogaea.* L) .16S rRNA sequence obtained was pasted into basic local alignment search tools (BLAST) in NCBI website and compared with available nucleotide sequences in Rhizobiaceae database to identify the closest homologs of the sequence. Isolate from Jar bahaushiya local variety was 99.15% similar with *Bradyrhizobium elkani, Mesorhizobium loti, Allorhizobium vitis, Sinorhizobium melitoti*, *Agrobacterium radiobacter* and *Agrobacterium vitis* with a sequence length of 627bp.

Table 1: Morphological Properties of Rhizobia isolates from Groundnut Varieties (*Arachis hypogaea* L)

Groundnut varieties	Colony shape	Colony color	Colony margin	Colony surface	Colony characteristic	Transparency
Samnut 23	Rod	Whitish	Entire	Smooth	Mucoid	Translucent
Samnut 24	Round	Whitish	Entire	Smooth	Mucoid	Translucent
Samnut 26	Round	Whitish	Entire	Smooth	Mucoid	Transparent
Samnut 27	Rod	Yellow	Entire	Smooth	Mucoid	Translucent
Samnut 28	Round	White	Entire	Smooth	Mucoid	Translucent
Samnut 29	Rod	White	Entire	Smooth	Mucoid	Transparent
Maibargo	Round	Yellow	Entire	Smooth	Mucoid	Transparent
Farar bahaushiya	Rod	Whitish	Entire	Smooth	Mucoid	Transparent
Sabeya	Rod	Yellow	Entire	Smooth	Mucoid	Translucent
Ex -Dakar	Round	Yellow	Entire	Smooth	Mucoid	Translucent

Table 2: Biochemical Characteristics of Rhizobia strain from the root nodules of Groundnut Varieties (*Arachis hypogaea* L)

Groundnut Varieties	Gram reaction	Reaction with yeast extract agar	Congo red reaction	yeast extract agar and bromothymol blue	Salt tolerance % (w/v)	Growth pH	Growth Temp (°C)	Cultured in peptone glucose agar plate	**Starch hydrolysis test**
Samnut 23	Negative	White colonies	Deep black	Yellow	0.5	5.5	22	+	Clear zone
Samnut 24	Negative	White colonies	Pale Pink	Yellow	1.5	5.5	27	_	Clear zone
Samnut 26	Negative	White colonies	Pink	Blue	1.00	8.5	35	_	Clear zone
Samnut 27	Negative	White colonies	Deep black	Yellow	0.5	5.8	22	+	Clear zone
Samnut 28	Negative	White colonies	Pale Pink	Yellow	2.00	6.0	35	_	Clear zone
Samnut 29	Negative	White colonies	Pale Pink	Yellow	1.5	5.2	27	_	Clear zone
Maibargo	Negative	White colonies	Pale Pink	Blue	0.5	8.5	20	_	Clear zone
Farar bahaushiya	Negative	White colonies	Deep black	Yellow	1.00	5.8	20	+	Clear zone
Sabeya	Negative	White colonies	Pink	Yellow	2.00	5.0	27	_	Clear zone
Ex-Dakar	Negative	White colonies	Deep black	Blue	1.00	9.5	27	+	Clear zone
S. E ±					0.17	0.52	1.71		

Key: No – Growth + Growth

Table 3: Result of Molecular Characterization of Rhizobia Strains isolated from Groundnut (*Arachis hypogaea. L*)Varieties.

	STRAINS	**HOST PLANT**	**SIMILARITY**
	Bradyrhizobium elkani, Mesorhizobium loti, Allorhizobium vitis, Sinorhizobium melitoti, *Agrobacterium radiobacter* and *Agrobacterium vitis*	Jar bahaushiya	99.15%

DISCUSSION

Morphological and biochemical tests are one of the main criteria for primary identification and characterization of rhizobia isolates (Somasegaran *et al.,* 1994). The results were inconformity with the result obtained by Agah *et al.* (2016) which showed that, the colonies of Rhizobia species isolated from root nodules of *Arachis hypogaea* L. and *Telfairia occidentalis* were mucous, rod-shaped, raised with smooth edges when observed under low power microscope. These results concur with those of Zilli *et al.* (2004) who reported that isolates showed mucous production that ranged from high to intermediate (Giongo, 2010). Batista *et al.* (2007) noted increased production of mucus in isolates of *Bradyrhizobium* as a mechanism of adaptation to acid soils. Sayyed *et al.* (2011) also reported that mucus protects rhizobia and serves as a potential energy reserve under water-deficient conditions. Therefore, mucus production by all rhizobia isolates in this study is the quality of the isolates associated with survival and nodulation.

The morphological characterization was to some extent in line with the result obtained by Gilbert *et al.* (2018) in which the colony color varied with milky white, cream white, cream yellow and watery colonies being observed which were either opaque or translucent with either firm gummy or smooth mucoid texture. The result of research was in line with the result obtained by Gilbert *et al.* (2018), who reported that all isolates had an entire colony margin. Based on the morphological characters studied, these isolates were identified as *Rhizobium* as observed by Somasegaran and Hoben (1994).

The results of the research did not agree with the finding of Mulugeta *et al.* (2013) which showed the rough colony morphology, as against smooth colony which may belongs to *R. leguminosarum* (Silva, *et al.,* 2003

The results were in conformity with that of (Agah *et al.*,2016) who revealed that all the isolates were gram negative. But did not agreed with result of Alemayehu (2009) who reported that all the strains showed growth in three days and turned the yeast extract mannitol agar media containing bromothymol blue to yellow color confirming that all the isolates were fast growers and acid producers. The results of biochemical characterization were in conformity with the result obtained by Tulu *et al.* (2018), who discovered that isolates grew at pH values within 5.5-10.5, tolerate high pH, with optimum growth temperature which ranged between 20-36^{o}C and can grew at salt concentration as high as 2%.

These findings were in closed agreement with Javed and Asghari (2008) who have previous characterized *Rhizobium* from soil and root nodules of Groundnut with similar positive biochemical tests. Similarly, Oblisami, (1995) studied the nodulation pattern in legume plants by screening through the same tests and reported similar results. Singh *et al*., (2008) characterized Rhizobium strain from the roots of Groundnut bacterial species.

The results also agreed with the findings of Gilbert *et al.* (2018) who reported that there was absence of Rhizobia growth when cultured in peptone glucose agar plates, incubated at 28°C for 4 days in the dark, this was a clear indication of the presence of *Rhizobium*.

The result was inconformity with that of Gilbert *et al.* (2018) and that of Devendra *et al.*, (2020) who discovered that, the isolates were Gram-negative, rods in shape and poor absorption of Congo red dye of the isolates on YEMA-CR further reinforced that the isolates were rhizobia (Beck *et al.*, 1993). Their growth within 3–5 days and the color change on YEMA-BTB from deep green to yellow was not in conformity with some of the results of the present work in which some rhizobia isolates turned yellow color with yeast extract and bromothymol blue. This suggested that some of the isolates were fast growers and could probably fall under the genera *Rhizobium, Allorhizobium, Sinorhizobium and Mesorhizobium* (Al-mujahidy *et al.,* 2013).

The result was also in conformity with the result obtained by Damaris *et al.* (2017) in which most of the isolates cultured on YEMA medium containing Congo red dye produced colonies that were pale to pale pink indicating that the isolates did not absorb the dye when incubated in the dark. The inability of the isolates to absorb Congo red dye is a distinctive character of Rhizobia. Strain of rhizobia do not absorb Congo red dye or may absorb little amount to give a pale pink appearance (Somasegaran *et al.*,1984). The result also agreed with the finding of Devendra *et al.* (2020) in which most of the strains did not absorb the Congo red dye from the YEMA media which is a distinctive characteristic of Rhizobia (Hewedy *et al.,*2014).

Some of the results were in conformity with the research of Agar *et al.(*2016) which revealed that after incubation for a week at 28 °C on YEMA with BTB, all the strains were found to be slow growing that showed an alkaline reaction in the medium as the dye turned to blue color. The Rhizobia strains that modulate groundnut roots

have been routinely considered as *Bradyrhizobium* spp., comprising slow growing strains capable of modulating several legumes.

Most of the results were in conformity with the research conducted by Singleton *et al. (*1984; Halima *et al. (*2012; Sarah. *et al.* (2015) in which most of the isolates tolerated high concentration of salt (2% NaCl) and were able to survive and tolerated temperature within 20-37°C, with a range of pH from 4.8-8.8. It was in conformity with the research of Zahran *et al.* (2012) which indicated that all rhizobia strains growth at 6- 8 pH. Positive results were obtained from the starch hydrolysis assay. On subjecting inoculated plates to iodine test, clear zones around the colonies were seen in the rhizobia isolates and the colonies turned yellow in appearance, which is in line with the research of De Oliveira *et al.* (2007) who observed that Rhizobia strains can utilize starch obtained from different sources. The result of molecular characterization of rhizobia strains isolated was in line with that of Tope *et al.* (2023) who investigated that the root of Bambra Groundnut is mostly associated with *Bradyrhizobium spp* using 16S rRNA sequence. It also agreed with that of Halima *et al.* (2012) who revealed that there was a great genetic diversity among the 17 Rhizobia strains studied. Indeed, sequence analysis of 16S rDNA and subsequent Blast analyses indicated that 12 strains had 98 to 100% similarity with Rhizobium sp., 2 strains were classified as *R. leguminosarum*;

. The result of the research also was not in line with the investigation of Özdoğan (2023) in which Rhizobia isolates were identified as *Agrobacterium tumefaciens*, and two isolates (F4DC and F6DC) were identified as *Rhizobium gallicum* at molecular level.

CONCLUSION

From the above result, it can be inferred that to identify rhizobia strains which are suitable for bioinoculant production, characterization of rhizobia is a prerequisite. *Rhizobium* inoculant improves crop yield, eco-friendly, increases crop yield and nutrient availability, low cost, prevent plant pests and diseases, bio remediation, increase resistance to water stress, mitigate or reduce greenhouse gas emission, land sustainability and improve soil quality, it can be used to substitute chemical fertilizer which is costly, lead to soil and land pollution, and health hazards.

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