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IDENTIFICATION OF RHIZOBIA ISOLATES OF SOME SELECTED GRAIN LEGUMES AT BAUCHI, GUINEA SAVANNA OF NIGERIA

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ABSTRACT

Cell colonies were extracted from nodules collected from some legumes grown in 2004, 2005 and 2006 rainy seasons. The legumes include cowpea*(Vigna unguiculata* L. Walp*), groundnut(Arachis hypogea* L.*) and soybean (Glycine max* L. Merill*)* treated with mineral nitrogen and phosphorus fertilizers at different levels, were laid out in a randomized complete block design (RCBD) replicated three times. The isolates' from the nodules were subjected to morphological, gram stain and biochemical identification. Plate cultures of the isolates revealed a morphological characteristic of rhizobia cells in a selective growth media of yeast mannitol agar (YMA) incubated at room temperature at 32^{o}C for 72hours. Morphological characteristics shows visible colonies appeared 2-4mm in diameter, circular, colourless, pulvanatate, raised elevation, smooth edge and surface, translucent viscous, musky odour. The cell morphology indicated bacterial cells were rods in chain, and dense clumps that stained gram negative by the appearance of faint pink red colour of the rod membrane walls. Inside the rods, there were small rounded granules that stained a dark purple. The biochemically identification of the colonies showed cells appeared smooth, white and raised colonies with a red halo streaked in a yeast mannitol agar supplemented by 2% (w/v) urea and 0.012% phenol. Rhizobia cells were also observed to appear smooth, white and raised colonies in 2days of incubation, with a red halo evidence of urea degradation confirmed rhizobia isolation. Yeast mannitol agar adjusted with dilute acid to 5.5 and with NaOH to 8.5 shows isolates suspected to be rhizobia appeared smooth, white, glistened and raised colonies of 2-4mm in diameter confirmed rhizobia presences. The isolated bacterial cell were also tested on the ability to grow in the presence of KNO_3 in a yeast mannitol agar under anaerobic conditions, the appearance of raised, white colonies of 2-3mm in diameter confirmed the presences of rhizobia. The ability of rhizobial strain to tolerate NaCl were tested on tryptone yeast extract agar supplemented with 0, 1, 2 and 3% (w/v) NaCl, colonies appeared raised white, smooth and glistened 2-4mm in diameter confirmed the presences of rhizobia. Furthermore, suspected rhizobia strains tested for antibiotic resistance in a yeast mannitol agar was supplemented with 3mg/ml streptomycin, the colonies appeared raised, white and glistened confirmed as rhizobia cells.

KEYWORDS*:* Identification, rhizobia isolates, grain legumes, Bauchi, Guinea Savanna, Nigeria

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INTRODUCTION

Rhizobia are nitrogen fixing bacteria that established an endosymbiotic association with legumes by the formation of specialized organs called nodules. Rhizobia are aerobic to microphilic, motile gram negative bacilli, with size ranging from 0.5-0.9 x 1.2-3µm, chemoorganoheterotrophic organism with sugar as their carbon source, GC content of 59-64% usually found in soil or root nodules of legumes (Singleton, 1999). They show optimum growth at

growth at temperature of 20-28%, with little growth or even death occurring at temperature above 40^{o}C (Vincent, 1977). This mutualistic relationship can be affected by the availability and unavailability of mineral nutrient most especially nitrogen and phosphorus, as reported by Pieternel and Jos (1995) that under conditions of soil nitrogen limitations, rhizobia elicit on their host the formation of specialized organs call nodules, which enable them to convert atmospheric nitrogen into ammonia, which is used by the plants as a nitrogen source. Furthermore, many of the legumes can obtain much of their N requirements through symbiotic N-fixation only if the root nodules are formed by effective strains of rhizobia. Phosphorus is an essential element for the growth of rhizobium species (Kamtin and Peter, 1987) and is one of the most deficient nutrients in highly weathered soils of the guinea savanna, containing high proportions of amorphous clays and/or iron and aluminium oxides. Since solution phosphates concentrations of most soils are less than 1µm, rhizobium strains having low tolerance to phosphate-deficient environments may have difficulty in nodulating legume grown in such soils. This study was carried out to identify the cells isolates from the nodules of these legumes, so as to enhance their capabilities in fixing atmospheric nitrogen into soils of guinea savanna of Nigeria.

MATERIALS AND METHODS

Sample Collection and Analysis

Nodules were collected from legumes grown in 2004, 2005 and 2006 cropping seasons. The legumes include cowpea (*Vigna unguiculata* (L.) Walp), groundnuts (*Arachis hypogea* (L.) and soybean (*Glycine max*(L.) Merill) treated with mineral nitrogen and phosphorus fertilizers at different levels. The treatments were laid out in a randomized complete block design (RCBD) replicated three times. The nodules obtained were surface sterilized with 95% ethanol for 40 minutes and then soaked in 0.1% sodium hypochlorite for 3-4 minutes. It was rinsed repeatedly with sterile distilled water (Vincent 1970, Esuobu, 1994 and Tas *et al* 1996). One gram of nodules per treatment was crushed in sterile crucibles using pistil and mortar. The completely crushed nodules were mixed with 10ml sterile distilled water in the crucibles, agitated and 1ml aliquot of the solution was collected and diluted in 3, 4 and 5 serial dilutions at 2, 4 and the rest of the weeks after sowing respectively. The dilutions were cultured in duplicate by pour planting into the selective medium of yeast mannitol Agar (YMA) and incubated aerobically at room temperature of 30°C – 32°C for 2-5 days, uncultured media were also incubated. Visible colonies were observed after 48-120 hours of incubation. The yeast mannitol agar was constituted as reported by Boonkerd and Weaver (1982), Weaver and Frederick (1982) and Tas *et al.,* (1996).

Morphological identification of isolates was based on growth on selective medium of Yeast mannitol agar (YMA), duration of incubation before the appearance of visible colonies (diameter and size ranging between 2-4µm), on Yeast mannitol agar (within 2-5 days at 25-30°C), cultured characteristics on Yeast mannitol agar and gram stain as described by Jordan (1984), and Weaver and Frederick (1982).

Biochemical identification of isolates as rhizobia cells was done using urea hydrolysis and growth in the present of 8% KNO_3 as described by Lindstrom and Lehtomaki (1988), growth in acid and alkaline medium as described by Zhang et al. (1991),sodium chloride tolerance as described by Brockman and Bezdicek (1989) and antibiotic resistance as described by Danso and Alexander (1974).

RESULTS

Identification of the Isolates

1 Morphology of rhizobia cell colonies on Yeast Mannitol Agar plate from nodules of the three legumes incubated at 31°C for 2 and 5 days after surface culture

Colonies morphology of the rhizobia from the crushed root nodules of cowpea, groundnut and soybean incubated on Yeast mannitol Agar plate at an average temperature of 31°C for 2 and 5 days is summarized in Table 1. The plates were 50% covered with a solid mass of the rhizobia species. After 48 and 120 hours incubation, visible colonies appearing 2-4mm in diameter, circular, colourless, (same as colour of the agar) pulvanate, raised elevation smooth edge and surface, translucent viscous, musky odour and have large areas of run on colonies that look like one large colony with many small tiny white round ones in linear patterns on the yeast mannitol agar.

2 Gram stain morphology of rhizobia cell colonies on Yeast Mannitol Agar plate of three legumes incubated at 31°C for 2 and 5 days after surface culture.

The cell morphology of the rhizobia from an isolated colour is presented in Table 2. The bacteria cells were rods, in chains, and dense clumps that stained gram negative as indicated by the faint pink red colour of the rod membrane walls. Inside the rods, there were small rounded bodies or granules that stained a dark purple. There were three to five granules inside the rod cells. These rods were wide and more irregular in shape and many were curved.

3 Biochemical identification of rhizobia cell colonies on Yeast Mannitol Agar plate of three legumes incubated at 31°C for 2 and 5 days after surface culture

Colonies suspected to be rhizobia isolates were identified biochemically using Hydrolysis of urea, growth in acid and alkaline medium, growth in the presence of KNO_3, sodium chloride tolerance and antibiotic resistance and it was observed that the colonies displayed a characteristics appearance of a rhizobial cell in the various test used Table 3.

DISCUSSION

Identification of the Isolates

The identification of the rhizobium species was on growing media selective to the rhizobium. The rhizobium agar is specie specific with yeast extract to provide a source of organic nitrogen and mannitol as the carbon source in the growing media. One colony plate was examined for the presence of rhizobium species. The most dominant type of colony was a large mass of white that covered more than 50% of the plate, with large patches of single colonies of the same morphology. The colony size of 2-4mm, circular in shape, colourless, raised, smooth, glistening with musky odour. The colony morphology of the rhizobium species tends to produce excessive amounts of slime, which is extracellular carbohydrate, when grown on a media that is nitrogen limited and contains mannitol as source of carbohydrate (Broughton *et al*., 2000).

The gram stains of the isolated colonies indicated the presence of bacteria cells that have the characteristic cell morphology of the rhizobium species. The rods were rectangular, straight and with blunt rounded ends and stained gram negative. The rods were grouped in pairs, chain and dense clusters. In addition, they contained 3-5 small dark staining round granules. The cell morphology of the rhizobium species are characterized as blunt rod shaped cells in pairs, chains and in groups. They are gram stain negative and appeared banded due to the presence of β-hydroxybutyrate granules (Broughton *et al*., 2000).

The combination of the cell morphology of the growth of the colonies on the selective media colony morphology and gram stain provides sufficient evidence to demonstrate the successful isolation of a rhizobium species (Noble, 1998). However, under these experimental conditions it is inclusive to state that the rhizobium species fix atmospheric nitrogen because the rhizobium species was grown in a medium that provided organic nitrogen in the form of the yeast extract. Rhizobium species can only fix atmospheric nitrogen under microaerophilic conditions such as inside the root nodule of leguminous plant (Royal, 1997).

The biochemical identification of the isolates showed that discrete colonies streaked in a yeast mannitol agar supplemented by 2% (w/v) urea and 0.012% phenol appeared smooth, white and raised colonies in 2days of incubation, with a red halo evidence of urea degradation confirmed rhizobia isolation. This agrees with the findings of Lindstrom and Lehtomaki (1988) who reported that rhizobia contain the enzyme urease which is capable of hydrolysing nitrogen to release ammonia during N-fixation. Yeast mannitol agar adjusted with dilute acid to 5.5 and with NaOH to 8.5 shows isolates suspected to be rhizobia appeared smooth, white, glistening and raised colonies of 2-4mm in diameter confirmed rhizobia presences. The appearance of the colonies conforms to the report of Zhang *et al*. (1991) that rhizobia species strive in both acid and alkaline medium of 5.5 to 8.5. The isolated bacterial cell were also tested on the ability to grow in the presence of KNO_3 in a yeast mannitol agar under anaerobic conditions, the appearance of raised, white colonies of 2-3mm in diameter confirmed the presences of rhizobia. The finding in this study is in conformity to the report of Lindstrom and Lehtomaki (1988) that in an anaerobic environment rhizobia

cells hydrolyses nitrogen from nitrate containing compounds. The ability of rhizobial strain to tolerate NaCl were tested on tryptone yeast extract agar supplemented with 0, 1, 2 and 3% (w/v) NaCl, colonies appeared raised white, smooth and glistening 2-4mm in diameter confirmed the presences of rhizobia. Beringer (1974); and Brockman and Bezdicek (1989) reported the ability of rhizobia cells to grow in salt environment. Furthermore, suspected rhizobia strains tested for antibiotic resistance and a yeast mannitol agar was supplemented with 3mg/ml streptomycin the colonies appeared raised, white and glistening confirmed as rhizobia. The evidence of growth is in conformity to the report of Danso and Alexander (1974) that rhizobia being soil bacteria have the ability to resist antibiotic secretions from soil fungi.

CONCLUSION

It is evident that the isolates collected from the root nodules of these legumes have shown clear characteristics of bacteria cells. The culture of the isolate was carried out in a Yeast Mannitol Agar (YMA) which is a selective growth media specifically for rhizobia species. It can be concluded that the isolates shows the presence of active rhizobia cells in the soil. Hence conscious effort is needed to enhance the performance of these legumes in order to increase their potentials for harnessing indigenous rhizobia species for sustainable soil productivity in Bauchi State.

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Table 1: Morphology of rhizobia colonies on Yeast Mannitol Agar plate of the three legumes incubated at 31°C for 2 and 5 days after surface culture.

Characteristics	Description
Size	2-4mm diameter
Shape	circular
Pigmentation	colourless, same colour as the agar
Elevation	pulvinate, raised
Edge	smooth
Surface	smooth
Under reflected light	opalescent, glistening
Consistency	viscous
Odour	musky

Table 2: Gram stain morphology of rhizobia cell colonies on Yeast Mannitol Agar plate of three legumes incubated at 31°C for 2 and 5 days after surface culture.

Characteristics	Description
Shape	rectangular rods
Axis	straight, bent or curved
Ends	rounded
Grouping	paired, chains and dense clusters
Gram stain	negative
Other inside the	3-5 round purple granules rods. Some may be parts of broken rods appear bloated and twisted.

Table 3: Biochemical identification of rhizobia cells colony on Yeast Mannitol Agar plate of three legumes incubated at 31.5°C for 2 and 5 days after surface culture.

Biological test	Description
Hydrolysis of Urea	raised, smooth and white colonies with re halo evidence of urea degradation
Growth in Acid and Alkaline medium	raised, smooth and white colonies
Growth in the presence of KNO_3	raised, smooth and white colonies
Sodium chloride tolerance	raised, smooth and white colonies
Antibiotics resistance	raised, smooth and white colonies