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Full Length Research Paper

Screening of bacteria isolated from the rhizosphere of maize plant (*Zea mays L.*) for ammonia production and nitrogen fixation

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Eleven (11) rhizobacteria identified as *Azospirillum* sp. isolated from the rhizosphere of maize plants grown in Ibadan, Oyo State, Nigeria were evaluated for ammonia production and nitrogen fixation. The micro-Kjeldahl method was used for the screening of the isolates for nitrogen fixation. Nitrogenase activity ranging from 1.20 to 10.60% was detected in seven of the eleven isolates. Results show that treatments with the application of organic fertilizers enhanced bacterial population and also showed higher nitrogenase activity in rhizosphere soil compared to inorganic fertilizer and control treatments. This showed that organic manure would be a better alternative to chemical fertilizers in maize farming. It was also observed from this study, that *Azospirillum* possess high nitrogenase activity allowing for the possibility of using this bacteria as a biofertilizer to improve soil fertility for improved and efficient farming.

Key words: Rhizobacteria, rhizosphere, nitrogen fixation, nitrogenase activity, biofertilizer.

INTRODUCTION

The rhizosphere is the region of soil surrounding plant roots which is characterized by enhanced microbial activities. This region supports a large and active microbial population including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Rhizobium* and *Serratia* (Kumar et al., 2012); which are capable of exerting beneficial, neutral and detrimental effects on the plants. These organisms can increase soil productivity through the improvement of soil fertility, production of plant growth hormones, phosphate solubilization, nitrogen fixation etc. bio-composting and biodegradation (Ahemad and Kibret, 2014; Kumar and Gopal, 2015). These groups of plant growth promoting rhizobacteria (PGPR) have been used extensively as bio-fertilizer thus reducing the over-reliance on chemical fertilizers, which have various drawbacks and disadvantages (Ahemad and Kibret, 2014; Verma et al., 2013).

According to Richard and Ogunjobi (2016), soil nutrient depletion has been a major challenge in Nigeria as a result of continuous cultivation of soils without adequate addition of external inputs. This has brought about the risk of continuous decline of soil nutrients, hence, the

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nutrients are replenished through the use of organic or mineral fertilizers which are partially returned through crop residues or through traditional fallow systems. For higher productivity in agriculture, heavy doses of fertilizers and other agrochemicals are applied to the soil to increase crop yield. These synthetic fertilizers increase plant yield but with some accompanying side effects like disrupting the microbial ecology of the soil and their deleterious effects on the environment (Benton, 2012). The application of chemical fertilizers could enhance the nutrient balance of soils, leading to increase in crop yields, but its continuous use is hazardous both to human health and the environment (Glick, 2003) as it may cause plant toxicity (Nazar et al., 2012) while the accumulation of trace metals in plants could pose a possible health risk to humans when consumed (Khan et al., 2015; Roy and McDonald, 2015). In a study by Geisseler and Scow (2014), the long term use of mineral nitrogen fertilizer caused an increase in soil pH, osmotic potential and ammonia concentration which eventually resulted in a sharp drop in the soil microbial biomass. The negative effects of chemical fertilizers could be avoided by using organic fertilizers which have a positive effect on the PGPR as reported by Richard and Ogunjobi (2016). Organic manure applications improved soil physical properties through increased soil aggregation (Zhang and Fang, 2007), decrease in the volume of micropores while increasing macropores (Hati et al., 2006), increased saturated hydraulic conductivity (Ndiaye et al., 2007) and water infiltration rate (Rasool et al., 2007).

Nitrogen (N) is one of the most important mineral nutrients required in large quantity by plants and whose deficiency mostly limits plant growth and development. Most tropical soils however are deficient in available nitrogen. Moreover, in ecosystems with low N inputs and without any form of soil amendments by humans, nitrogen is found in the gaseous state, a form which is not usable by plants and animals (Calvaruso et al., 2006). The plants get nitrogen, mainly from the application of nitrogen fertilizers, of which 50% are utilized and the rest lost through leaching, denitrifiction and volatilization (Saikia et al., 2004). The plants are unable to utilize the molecular nitrogen unless it is converted to ammonia through the process of biological nitrogen fixation (BNF), which is carried out by microorganisms, especially the rhizosphere bacteria. This presents an inexpensive, environmentally friendly and sustainable approach to crop production and constitutes an important plant growth promotion scenario (de Bruijn, 2015). Nitrogen fixation is the second most important process after photosynthesis which has significant function in crop production (Reddy et al., 2016). A large number of diazotrophs, such as *Azospirillum, Gluconacetobacter diazotrophicus, Azoarcus, Beijerinckia, Enterobacter, Klebsiella, Pseudomonas, Azorhizobium, Herbaspirillum* and *Azotobacter* inhabit both root and stem of plants and they are more effective than their rhizospheric counterparts in terms of benefiting their host through nitrogen fixation as they can provide fixed nitrogen directly to their host (Cocking, 2003; Omer, 2017). Nitrogen fixation by *Azospirillum* sp. in association with grasses and other non-leguminous plants has been examined by researchers. *Azospirillum* is currently one of the most broadly studied and commercially employed PGPR as previous studies have emphasized its capacity of fixing atmospheric N_2, followed by benefits in promoting plant growth via synthesis of phytohormones (Fukami et al., 2018). Although it appears that *Azospirillum* lacks host specificity in the promotion of plant growth (Pereg et al., 2016), several studies evaluated its capacity to fix N_2 and to replace N-fertilizers when associated with grain crops such as maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), and rice (*Oryza sativa* L.) among others (Marks et al., 2015; Fukami et al., 2016, 2017; Pereg et al., 2016). The aim of this study was to isolate diazotrophs from the rhizosphere of maize and screen them for nitrogen fixing and ammonia production abilities.

MATERIALS AND METHODS

Experimental design

The experiment was carried out at the plant field of Department of Botany, University of Ibadan, Nigeria. The plot used for the experiment was divided into six blocks with four of the blocks (two each) treated with organic amendments, poultry litter and inorganic fertilizer (N:P:K 12:12:17). The last two blocks were used as the control block (without any treatment). Grains of maize variety BR9928DMRSR-Y obtained from International Institute of Tropical Agriculture (IITA), Ibadan headquarters were planted on the blocks for the experiment. Ten holes with three seeds each were planted per block.

Isolation and characterization of rhizosphere bacteria

Soil samples from the rhizosphere of maize plants in the experimental farm were collected at different growth stage from each treatment and placed in plastic bags for transport to the laboratory. Excess soil was shaken off and the soil strongly adhering to the roots were immediately used without drying for analysis as described by Basul et al. (2010). Sampling was done for a period of 56 days at an interval of 14 days. The rhizosphere soil samples were analyzed on Congo-Red medium using the pour plate technique for the isolation of Nitrogen-fixing bacteria. The isolates obtained were characterised using morphological, biochemical and sugar fermentation tests.

Determination of ammonia production

All the isolates were tested for the production of ammonia using the qualitative method of Ahmad et al. (2008). The development of a brown to yellow colour was indicative of ammonia production.

In-vitro screening for nitrogen fixing activity

Nitrogen free malate medium (Döbereiner et al., 1995) containing bromothymol blue (BTB) as an indicator, was used for the

Table 1. Distribution of bacterial isolates from rhizosphere soil.

Treatments used	No. of isolates	Isolate codes
Organic manure	4	OM1, OM2, OM3, OM4
Inorganic fertilizer	4	IN1, IN2, IN3, IN4
Control	3	CON1, CON2, CON3

preliminary screening of the isolates and were incubated at 37 and 50°C for 24 h. Isolates producing blue coloured zones were marked as nitrogen fixers.

Assay for nitrogen fixation

The efficiency of nitrogen fixing ability of the isolates were determined by micro-Kjeldahl analysis as described by Bergersen (1980) by inoculating the isolates in semisolid Nfb medium containing 0.05% of malate as carbon source and incubated at 32°C. Triplicates were maintained in each isolates. The percentage of N_2 in the sample was calculated with the formula below:

$$= \frac{\text{Sample titre} - \text{Blank titre}}{\text{Sample weight in g} \times 1000} \times \text{Normality of HCl} \times 14 \times 100$$

RESULTS AND DISCUSSION

Isolation strategies of nitrogen fixing bacteria

A total of eleven nitrogen fixing bacteria isolates were obtained from the different treatments of organic manure (OM), inorganic fertilizer (IF) and untreated control (CON) soil using Congo-Red medium during the 56 days of isolation. The distribution of the isolates is presented in Table 1. These isolates were initially considered as nitrogen fixers since they were able to grow on Congo-Red medium which is a selective medium for nitrogen fixers.

Enumeration of rhizobacteria

The total rhizobacterial Nitrogen fixing bacteria isolates within the rhizosphere of maize plant is shown in Figure 1. The rhizobacteria increased from 3.5×10^9 to 6.4×10^9 CFU/g in rhizosphere soil amended with organic manure; while that in soil amended with inorganic fertilizer increased from 4.1×10^9 to 6.0×10^9 CFU/g while that in the untreated control soil increased from 3.0×10^9 to 4.8×10^9 CFU/g from day 14 to day 56.

Morphological and biochemical identification of nitrogen fixing isolates

The results of the morphological and biochemical tests are presented in Table 2 and all the isolates were identified as *Azospirillum* sp.

Nitrogen fixing screening of isolates

Nitrogen fixing ability of the eleven diazotrophs was measured using the micro-Kjeldahl method. Among the eleven isolates tested, seven isolates showed nitrogen fixing ability ranging from 1.20 to 10.60%. Among them, the maximum nitrogen fixing activity (10.60%) was recorded with *Azospirillum* sp. OM4 and minimum (1.20%) was recorded for *Azospirillum* sp. CON1. Among the 11 isolates, 5 isolates; *Azospirillum* sp. OM4, OM3, IN3, IN2 and CON3 fixed the highest amount of nitrogen such as 10.60, 8.80, 8.00, 6.20 and 6.00% respectively. Four isolates namely *Azospirillum* sp. OM1, OM2, IN4 and CON2 could not fix any nitrogen. Figure 2 presents the highest percentage of nitrogen fixed by the isolates from the different treatments during each sampling period (Day 14, 28, 42 and 56). For Day 14, only IN1 fixed nitrogen (1.60%). At Day 28, IN2 had the highest nitrogen fixed (6.20%). OM3 fixed the highest nitrogen with 8.80% at Day 42. Similarly, OM4 fixed the highest nitrogen that is, 10.60% at Day 56.

Figure 1 showed that increase in rhizobacterial count was directly proportional to increase in the duration of maize cultivation. This could be attributed to the fact that the soil is a suitable medium for the growth of environmental microorganisms because it is rich in nutrients. Furthermore, the treated soil samples recorded higher bacteria counts as compared to the control treatment and this might be a result of the additional nitrogen provided by the additives which the bacteria breakdown for plant use resulting to the release of more exudates and plant products for the diazotrophs, hence, increasing in rhizosphere bacterial biomass as reported by Das and Dkhar (2011). However, the soil sample treated with organic manure had larger bacteria pool than in the same soil receiving only chemical fertilizers just as reported by Islam and Weil (2002). Similar observations were also reported in organic recycling experiments by Chakrabarti et al. (2000) where soil receiving more organic matter harbored higher levels of bacteria corresponding to higher microbial activity (Mäder et al., 2002). The bacterial count for treatments with inorganic fertilizer were initially higher, nevertheless, the bacterial count for the organic manure treatments increased gradually and surpassed the inorganic fertilizer treatment

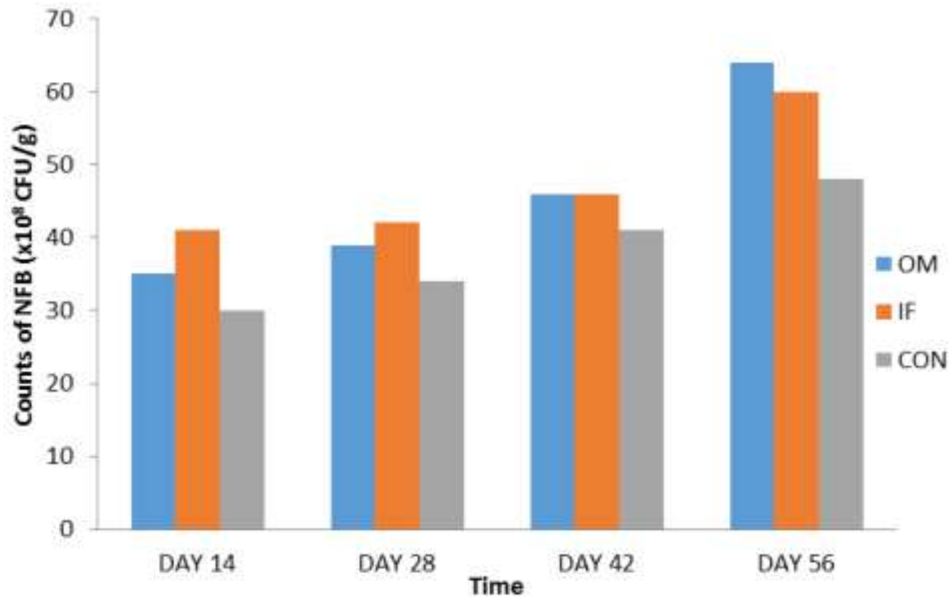

Figure 1. The count of nitrogen fixing bacteria in the rhizosphere of maize.

Table 2. Morphological, biochemical characterization and sugar fermentation of isolates.

Isolates	Morphology	Gram's Reaction	Catalase	Oxidase	Indole	Citrate	Motility	Methyl Red	Voges Proskauer	H_2S Production	Starch Hydrolysis	Urease	NH_3 Production	Glucose	Galactose	Sucrose	Fructose	Lactose	Maltose	Mannitol	Probable organism
OM1	R	-	+	+	-	-	+	-	+	-	-	+	+	-	+	-	-	+	-	+	*Azospirillum* sp.
OM2	R	-	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-	+	-	+	*Azospirillum* sp.
OM3	R	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	*Azospirillum* sp.
OM4	R	-	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	-	+	*Azospirillum* sp.
IN1	R	-	+	+	-	+	+	-	-	-	-	+	+	+	-	-	+	+	-	+	*Azospirillum* sp.
IN2	R	-	+	+	-	+	+	-	+	-	-	+	+	+	+	-	+	+	-	+	*Azospirillum* sp.
IN3	R	-	+	+	-	+	-	+	-	-	-	+	+	+	-	-	+	+	-	+	*Azospirillum* sp.
IN4	R	-	+	+	-	-	+	+	-	-	-	+	+	-	-	-	+	-	-	+	*Azospirillum* sp.
CON1	R	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-	+	-	-	+	*Azospirillum* sp.
CON2	R	-	+	+	+	-	-	-	+	-	-	+	+	+	+	-	-	-	-	+	*Azospirillum* sp.
CON3	R	-	+	+	+	+	+	-	-	-	-	+	+	+	-	-	-	-	-	+	*Azospirillum* sp.

eventually. This observation is in line with the study of Adegbidi et al. (2003) who reported that the release of nutrients from composts and processed organic manures are generally slower than in inorganic fertilizer. In this study, all the isolates were identified as *Azospirillum* sp. and they all produced ammonia which is an essential step in nitrogen fixation. This is in line with the report of Kanimozhi and Panneerselvam (2010) who isolated *Azospirillum* sp. that was able to fix nitrogen from a district in Thanjavur. This ability to fix nitrogen by *Azospirillum* is due to the possession of the nitrogenase enzyme which catalyzes the nitrogen-fixing reaction (Kaczmarek et al., 2018). As shown in Figure 2, of the eleven isolates screened, only seven were able to fix nitrogen. This is similar to the findings of Naureen et al. (2005) where nineteen out of thirty isolates showed nitrogenase activity ranging from 21.8 - 3624 nmol of C_2H_4 mg protein^{-1}h^{-1}. Reddy et al. (2016) had a similar

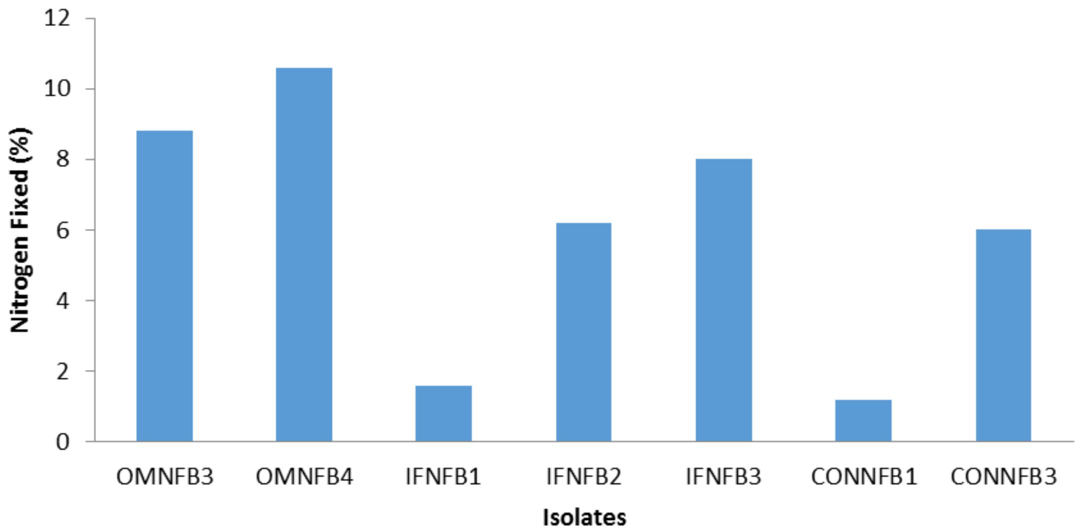

Figure 2. Percentages of nitrogen fixation by the obtained isolates (Key: OMNFM = OM, IFNFB = IN, CONNFB = CON).

report in which five of eleven isolates screened for diazotrophy were able to fix nitrogen. The variability of the nitrogenase activity of *Azospirillum* has been observed *in-vitro* by Han and New (1998) with Acetylene Reduction Analysis (ARA) varying from 0 to 155 nmol of C_2H_4 mg protein^{-1}h^{-1}, in pure cultures of *Azospirillum lipoferum* and *Azospirillum brasilense* obtained from soils of different regions as reported by Omer (2017). This agrees with the present study as it was shown (Figure 2) that large variability exists in the nitrogen fixing ability of *Azospirillum* isolates (1.20 to 10.60% nitrogen fixed). In their study, Omer (2017) selected two *A. brasilense* strains as the most efficient endophytic bacteria based on their activity in nitrogen fixation and indole-3-acetic acid production. Furthermore, Baskar and Prabakaran (2015) reported in the nitrogenase activity of *Azotobacter* and *Azospirillum* using the Acetylene Reduction Assay. These reports and the current study demonstrated that organic manure application supports/enhances the activity of *Azospirillum* in the rhizosphere of maize, which might be applicable to other cereal plants.

Conclusion

Inorganic fertilizer treatment has the ability to improve the nitrogenase activity of diazotrophs, but over time, their nitrogen fixing ability declines. On the other hand, organic manure treatments had the ability to improve the nitrogenase activity of diazotrophs at a gradual pace and this ability gets progressive over time. However, the duration of this study is not enough to conclude that long term exposure of inorganic fertilizer has total adverse effect on the nitrogenase activity of diazotrophs. We can, however, conclude that organic manure treatments had a positive effect on the microbial biomass and diazotrophic activities of rhizobacteria in non-leguminous plants such as maize. Therefore, organic manure treatment is a better alternative to chemical fertilizers in maize farming.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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