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Research Paper

Isolation, Molecular Characterization and Growth Promoting Activity of Free Living Diazotrophs Screened from Soils of Lesotho

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ABSTRACT

Thirty six free living diazotrophic isolates represented from different soil pH (5 to 7.22) of Lesotho were characterized. A nitrogen free medium was used to screen nitrogen fixing diazotrophs. Strains were classified into three groups as *Azospirillum*, *Azotobacter* and *Bacillus* spp based on their biochemical and molecular characteristics. All the diazotrophic groups showed some degree of nitrogenase activity when assayed using acetylene (C_2H_2) reduction assay and production of Indole Acetic Acid (IAA) when screened for PGPR traits. Despite the strain variability, significantly higher nitrogenase activity (190.20 nmol/mL^{-1}h^{-1}) was exhibited by strain NUL3-2 (*Azospirillum* spp), which in contrary exhibited lower amount of IAA production (0.032 mg ml-1) compared to strain MAF7-1 (0.448 mg ml-1) from the group of *Azotobacter* spp. Further evaluation of strains efficacy for plant growth promotion activity is commendable under greenhouse experimentation for potential use of these strains as biofertilizer.

Key words: Free living N_2 fixers, *Azosprillum* spp., nitrogenase activity, *Azotobacter* spp., *Bacillus* spp.

INTRODUCTION

Nitrogen (N), a constituent of amino acids and nucleic acids remains a growth limiting nutrient to all living things. The continual loss of nitrogen from the reserve of combined or fixed nitrogen by microbial denitrification, soil erosion, leaching, chemical volatilization and/or removal of nitrogen-containing crop residues from the land negatively affect soil fertility and crop production (Hubbell and Kidder, 2003). Nitrogen reserve of agricultural soils must therefore be replenished periodically in order to maintain an adequate or non-growth limiting level for crop production (Hubbell and Kidder, 2003).

The application of chemical fertilizers or urea may restore soil fertility and produce good yields. However, due to low efficiency of use which is mainly caused by NH_3^+ volatilization, denitrification (Bouwman et al., 2002), and losses from leaching (De Datta and Buresh, 1989; Bijay-Singh et al., 1995), only < 50% of the applied fertilizer is used by plants (Garabet et al., 1998; Choudhury and Khanif, 2001; Halvorson et al., 2002).

Biological Nitrogen Fixation (BNF) is an alternative technology to supplement or replace chemical fertilizer particularly to acidic soils (Jeyabal and Kuppuswamy, 2001). In legume plants, symbiotic nitrogen fixation accounts for 100 to 300 kg of nitrogen fixation/ha/year (Graham, 1998). The free-living non-symbiotic nitrogen fixing bacteria *Azospirllum* (aerobic) (Sumner, 1990**;** Saubidet and Barneix, 1998) and *Clostridium spp* (anaerobic) (Socolow, 1999) play an important role in non-legume plants crop farming.

The application of BNF offers an economically attractive and ecologically sound means of reducing external nitrogen input and improving soil quality (Cummings, 2005). Rhizosphere microbial communities also serve as Plant Growth Promoting Rhizobacteria (PGPR) to prevent the

deleterious effects of pathogenic organisms by the production of phytohormones, through phosphate solubilization and nitrogen fixation (Glick et al., 1999; Rodriguez and Fraga, 1999; Shridhar, 2012).

In Lesotho, seven of the ten benchmark soils are acidic and deficient in ammonium ion (NH_4^+) and assimilable inorganic phosphate ($H_2PO_4^-$) (Mosenene, 1999). Maize is one of the stable foods in Africa and grows best in neutral pH (Cummings, 2005). It requires 9 to 11 kg N to produce 1 ton biomass (Anuar et al., 1995). The restoration of soil pH by the addition of lime or fertilizers to acidic soils is often expensive and depletes soil organic matter (Wairiu and Lal, 2003). Therefore, the search for free-living nitrogen fixing bacteria is a viable option to provide sustainable solution for many agricultural practices.

In this study, the selection and *in vitro* evaluation of diazotrophs for their nitrogenase activity was conducted. Knowledge of the structure and diversity of Lesotho's soil microbial communities is important. The aim of this study was therefore, to isolate diazotrophs from Lesotho's soil and study their diversity using biochemical and molecular characterization.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from six different regions of Lesotho: Muela, Mahobong, Hlotse, Peka, Ha-Mafefooane and the National University of Lesotho campus. Areas were selected based on soil pH characteristics, which are from acidic to slightly alkaline. Soil samples were kept in the refrigerator at 4°C until used for isolation of nitrogen fixing bacteria.

Soil pH measurments

Soil pH was determined as per the methods described by Yang et al. (1999). One gram of soil was dissolved in 5 mL of sterilized distilled water, vortexed for 2 min and allowed to settle for 2 min. The pH probe was used to measure the pH in the middle of a suspension and data was recorded.

Isolation of diazotrophs

Nitrogen fixing bacteria was isolated using Nitrogen Free (NF) medium (Yang et al., 1999). Ten gram of soil sample was dissolved in 90 ml sterilized distilled water and one milliliter of the aliquot was serially diluted up to 10^{-7} in the respective 9 ml of Ringer's solution containing sterilized tube. A100 µl of serially diluted soil samples were spread plated on solid media. Each sample was done in duplicates and the plates were incubated at 25°C for 3 to 7 days. Any growth on the plate was regarded as positive results. The isolates were purified, sub-cultured and preserved on slant agar at 4°C in the fridge for subsequent trials.

Nitrogenase activity assay

Nitrogenase activity of the isolates was measured by acetylene reduction assay (Dilworth, 1966). The vial was filled with 10 ml of the isolates suspension and covered with rubber stoppers. Ten ml of air was removed from the vial and replaced with 10 ml of C_2H_2 (approximately, 10% of air volume) using a sterilized syringe and left to stand for 24 to 48 h at 28 to 30°C. Then, 0.2 ml of gas was removed from the vial and injected into gas chromatography column (Techcomp GC7890) to check the amount of C_2H_2 and C_2H_4. The following formula was used to determine the amount of nitrogenase activity:

$$N = (h\,x \times C \times V) \,/\, (hs \times 24.\,9 \times t)$$

Where, N = the concentration of C_2H_4 (nmol ml^{-1} h^{-1}); $h\,x$ = the peak value of the sample: C = concentration of standard C_2H_4 (nmol $ml^{-1}h^{-1}$); V = volume of the vial; hs = peak value of C_2H_4; t = the time taken to complete a reaction (h). Potential strains with high nitrogenase activity were further identified for their identity.

Indole Acetic Acid (IAA) production assay

The purified isolates with nitrogen fixing ability were tested for their ability to produce IAA according to Bric et al. (1991). The tests were done by preparing liquid Nitrogen Free medium with and without tryptophan (500 µg/ml). Aliquots of 100 ml of medium were inoculated with standard (10^8 cells ml^{-1}) concentration of potential strains and incubated on the shaker at 120 rpm and 25°C for 7days. Subsequently, two millilitres of the broth culture was centrifuged at 10,000 rmp for 15 min and one millilitre of the supernatant mixed with 100 µl orthophosphoric acid and Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) and allowed to stand for about 25 min for pink colour formation. The absorbance of the pink colour was measured spectrophotometrically at 530 nm and data recorded.

Biochemical and molecular characterization of isolates:

Morphological and biochemical characterization

The bacterial isolates were characterized by their cultural, morphological and biochemical characteristics and carbon

utilization (glucose, sucrose, lactose, mannitol, citrate oxidase and catalase) using standard methods (Cappuccino and Sherman, 2007).

16S rDNA extraction and analysis

An improved CTAB method (National Center for Biotechnology Information, 2014) was used to extract the DNA of six selected bacterial samples. The selection was made based on their activity in greenhouse experimentation. Ten milliliter of bacterial suspension was centrifuged at 8000 rpm for 10 min and the cells were re-suspended in TE-buffer (1.8 ml), 10% SDS (0.1 ml) and Proteinase K (10 µL of 20 mg/ml) solution and incubated at 37°C for 1 h. Under aseptic condition, the supernatant was transferred to a sterilized tube and a 0.3 ml of CTAB/NaCl (0.36 m/L) solution was mixed thoroughly and incubated at 65°C for 20 min. Equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and centrifuged at 6 000 rpm for 10 min. The supernatant was transferred to a new tube and eluted with equal volume of the same solution once and centrifuged. A 20 µL of *RNaseA* solution was then mixed with the DNA supernatant and incubated at 37°C for 1 h. The DNA solution was then centrifuged at 12 000 rpm until the DNA settled. Two milliliter of cold ethanol (70%) was added to the DNA extract after removing the aqueous phase and centrifuged at 10000 rpm for 4 min. The extracted DNA was then air dried at room temperature and stored at -20°C for further analysis. A 1% agarose gel electrophoresis was run with adjusted concentration of 400 ng/µL to measure the concentration of genomic DNA at OD_{600}.

PCR amplification and cloning

The 16S rDNA of the selected strains were amplified using forward primer (AgA gTT TgA TCC Tgg CTC Ag) and reverse primer (Aag gAg gTg ATC CAg CCg CA) (National Center for Biotechnology Information, 2014). The PCR products were recovered from agarose gel and purified by PCR product (TaKaRa Company, China). The pGEM – T Easy Vector System (TaKaRa Company, China) was used for the legation of DNA fragments. The legated products were transformed into *Escherichia coli* D5α cells. The white colonies and enzymatic restriction methods were used to screen for transformants for inserts. The clones which were positive were sent to Shanghai Boya Company for sequencing.

RESULTS

Isolation of diazotrophs

Thirty six potential free-living diazotroph strains were isolated from sample soils obtained from slightly acidic to slightly alkaline soil pH (5.32 to 7.22) , Lesotho (Table 1).

Nitrogenase activity

The concentration of ethylene (C_2H_4) was observed to be in the range of 97.01 to 190.20 nmol/mL^{-1} (Table 2). Strain NUL3-2 showed the highest reduction activity (190.2 nmol/mL^{-1}) as compared to the other strains (Table 2).

IAA Production

All strains showed some activity of IAA production; the range between 0.04 to 0.45 mg/ml (Figure 1). Four strains (PEK1-1, PEK5-1, NUL3-2 and NUL18-1) produced IAA less than 0.2mg/ml and two strains (MAF7-1 and MAF7-3) showed the production of IAA above 0.4mg (Figure 1).

Molecular characterization and identification of strains:

16S rDNA amplification and sequencing

The length of 16S rDNA for each strain amplified were found to be in the range of 1321 to 1410 bps (Figure 2).

Phylogenic analysis of 16S rDNA sequence

It was found that PEK1-1, MAF7-3 and NUL3-2 belonged to *Azospirillum*. There was a close similarity between PEK1-1 and *Azospirillum* sp (FN293046): MAF7-3 and *Azospirillum* sp (X92464): and NUL3-2 and *Azospirillum* sp TS11 (AB114192) (Figure 3a).

Strain MAF7-1 and NUL18-1 belong to the genus *Azotobacter* (Figure 2b) with close phylogenic relationship to *Azotobacter beijerinckii* G7 (AB429527), ICMP 8673 (EF100152) and ICMP 4032 (EF100151) respectively (Figure 3b). Strain PEK5-1 showed close phylogenetic relationship to the genus *Bacillus* sp. Strain TW116S (GU905015) (Figure 3c).

DISCUSSION

In this study, 36 strains of diazotrophs which were initially isolated and grouped into 6 operational groups were identified as *Azospirillum* (PEK1-1, NUL3-2 and MAF7-3); *Azotobacter* (NUL18-1 and MAF7-1) and *Bacillus* (PEK5-1) spp using16S rDNA sequencing and analysis.

The nitrogenase activity of *Azospirillum* spp was found to be the highest under aerobic conditions as compared to the

Table 1. Total number of isolated strains and soil pH.

Place	Soil sample	pH	No. of isolated strains	Strain code
Muela	MUE1	7.10	1	MUE1-1
Hlotse	HLO3	6.89	2	HLO3-1
				HLO3-2
Peka	PEK1	6.43	4	PEK1-1
				PEK1-2
				PEK1-3
				PEK1-4
Peka	PEK5	6.32	1	PEK5-1
Peka	PEK6	7.22	1	PEK6-1
NUL	NUL1	7.18	1	NUL1-1
NUL	NUL 2	6.13	3	NUL2-1
				NUL2-2
				NUL2-3
NUL	NUL3	6.93	2	NUL3-1
				NUL3-2
NUL	NUL11	6.59	2	NUL11-1
				NUL11-2
NUL	NUL15	6.67	1	NUL15-1
NUL	NUL18	6.60	1	NUL18-1
Mafefooane	MAF1	6.42	4	MAF1-1
				MAF1-2
				MAF1-3
				MAF1-4
Mafefooane	MAF3	5.32	5	MAF3-1
				MAF3-2
				MAF3-3
				MAF3-4
				MAF3-5
Mafefooane	MAF4	5.37	4	MAF4-1
				MAF4-2
				MAF4-3
				MAF4-4
Mafefooane	MAF5	6.88	1	MAF5-1
Mafefooane	MAF7	6.79	3	MAF7-1
				MAF7-2
				MAF7-3

The letters of the strain code are the first three letters of the name of the place where soil samples were collected, followed by the number of the soil sample, the last number represent the number of isolates. For example, the code HLO3-2, shows that the isolate was obtained from third soil sample from Hlotse, and it was the second isolate.

Table 2. Nitrogenase activity of the isolated strains.

Strains isolated	C_2H_4 concentration (nmol/mL^{-1})
PEK1-1	152±0.27^{b}
PEK5-1	97.01±0.11^{c}
NUL3-2	190.2±0.45^{a}
NUL18-1	120.18±0.14^{c}
MAF7-1	98.65±0.32^{c}
MAF7-3	118.5±0.12^{c}

Letters represent significance level at $P < 0.05$.

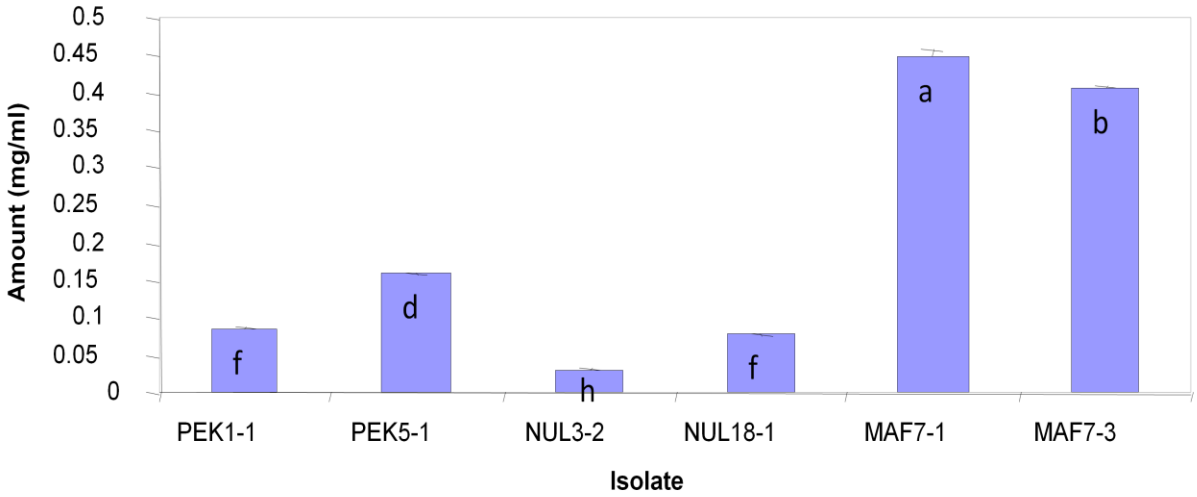

Figure 1. IAA production ability of diazotroph isolates. Mean values are expressed in bars. Bars designated wit the same letter are not significantly different according to Fisher's LSD test ($P< 0.05$) and t-grouping. Designated codes are referred as follows: Pek1-1, NUL3-2, MAF7-3 = *Azosprillium* spp.; NUL18-1, MAF7-1 = *Azotobacter* spp. and PEK5-1 = *Bacillus* sp.

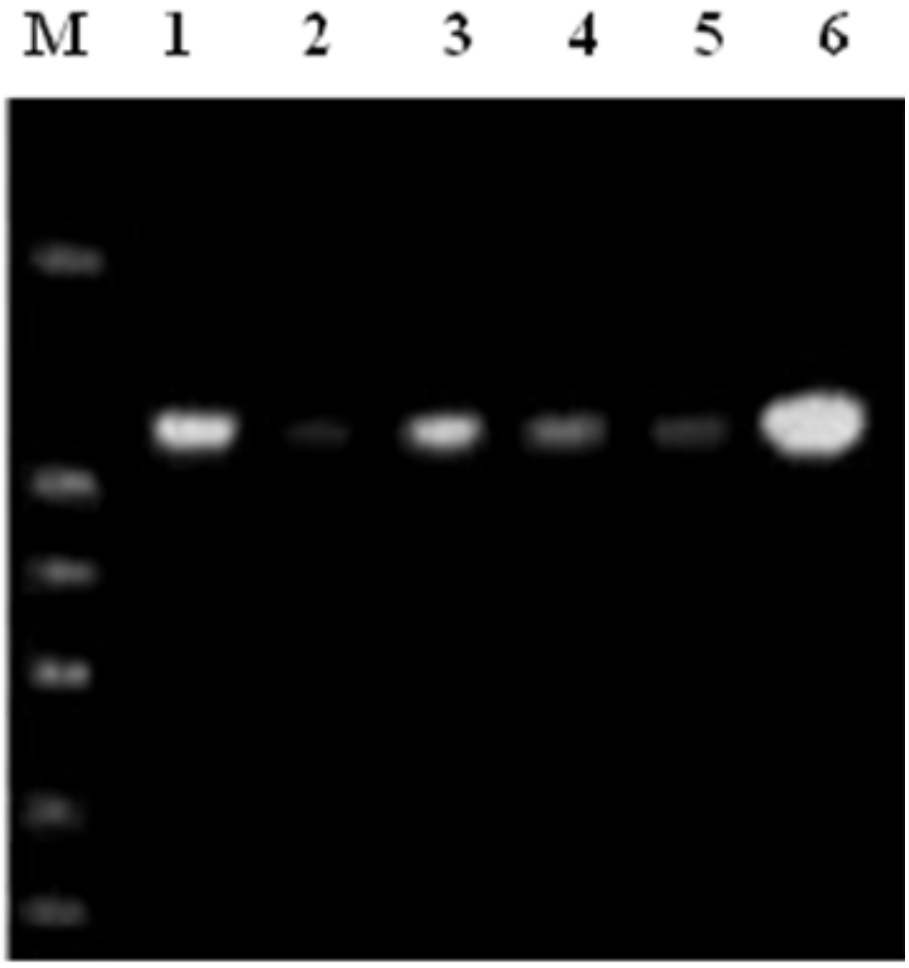

Figure 2. 16S rDNA PCR products. **M=** D2 000; 1= PEK1-1(1340bp); 2= PEK5-1(1286bp); 3= NUL3-2(1350bp); 4=NUL18-15(1321bp); 5= MAF7-1(1342bp); 6= MAF7-3(1410).

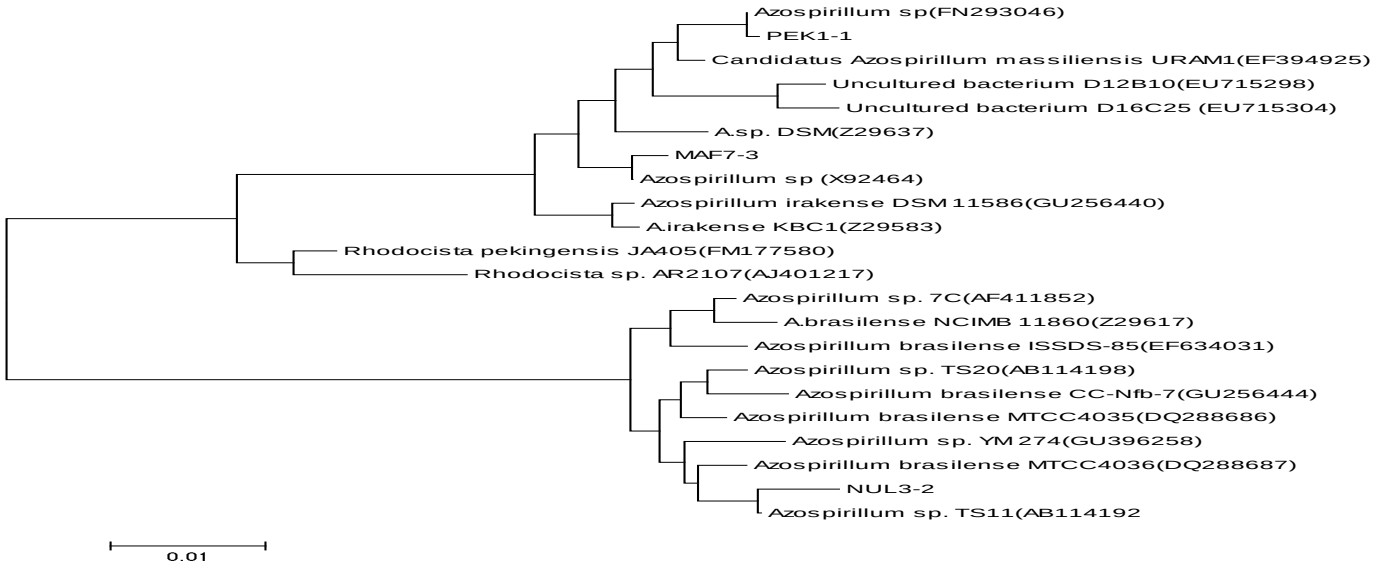

Figure 3a. Phylogeny of strain PEK1-1, NUL3-2 and MAF7-3.

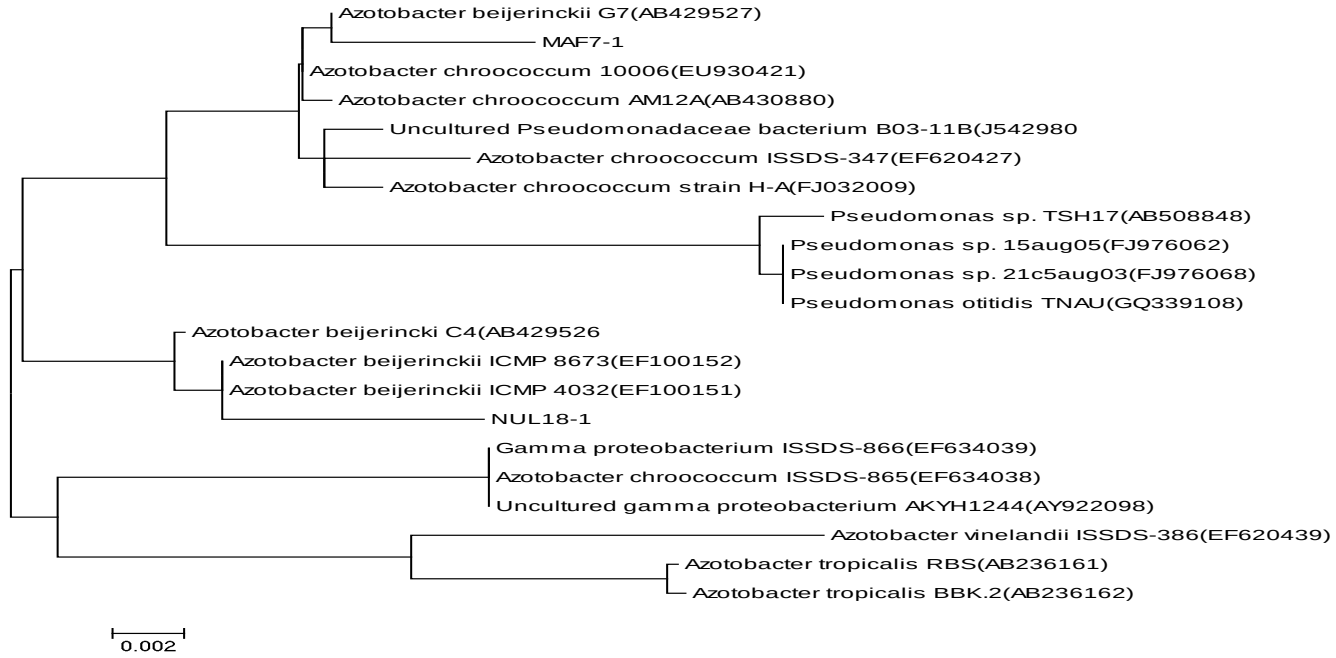

Figure 3b. Phylogeny of strain NUL18-1 and MAF7-1.

other groups of diazotrophs identified as *Azotobacter* and *Bacillus* spp, respectively. This could be due to microaerophillic nature of the *Azospirillum* spp that only fixes nitrogen under microaerobic conditions being energized by many organic acids and some monosaccharide and disaccharide molecules from the soil (Alvarez et al., 1996; Kanimozhi and Panneerselvam, 2010; Shridhar, 2012). It was assumed that inoculation with diazotrophic bacteria like *Rhizobium*, *Azotobacter* and *Azospirillum* enhanced the plant growth as a result of their ability to fix nitrogen; (Gadagi et al., 2004; Doroshenko et al., 2007) however, only rhizobia showed an increase in yield from nitrogen fixation (Kanimozhi and Panneerselvam, 2010).

Nitrogen fixing free living microorganisms has frequently been reported as plant growth promoters (Requena et al., 1997). In this study, although there is no direct correlation

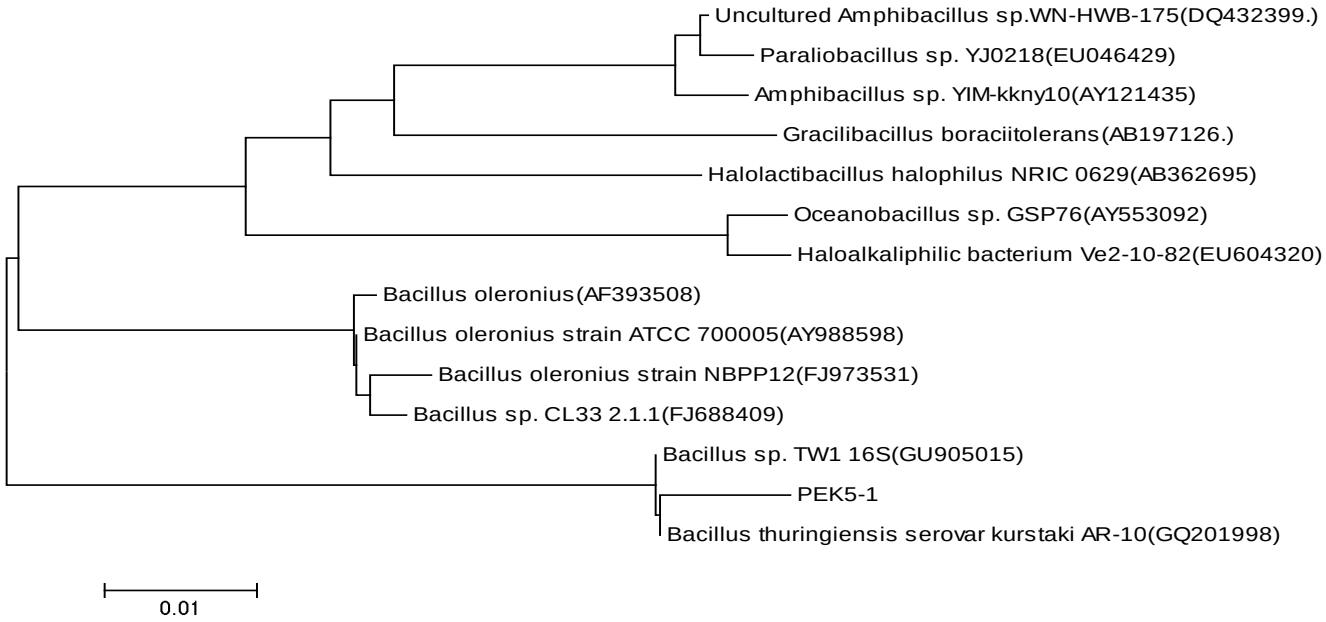

Figure 3c. Strain PEK5-1 phylogeny.

between the level of nitrogenase activity and plant growth promotion, the production of high concentration of IAA (Figure 1) has shown to have great influence on the germination of maize seed under laboratory experimental conditions (data not shown). This result is in line with the report of (Frankenberger and Arshad, 1995; Khalid et al., 2004; Fogaca and Fet-Neto, 2005), which reviled the production of IAA that has shown to promote root growth directly by stimulating cell elongation and division (Lambrecht et al., 2000).

In vitro evaluation of diaztrophic strains for antagonistic activity against *Fusarium oxysporum* showed suppressing activity of pathogen mycelial growth (data not shown) indicating their potential for disease management as (PGPR) and bacterial endophytes (Shridhar, 2012; Amaresan et al., 2014).

Conclusion

Significant level of nitrogenase activity was observed by strain NUL3-2 while strain MAF7-1 and MAF7-3 showed high efficiency in promoting maize germination. Although, there is no significant correlation exhibited between nitrogenase activity and plant growth promoting activity from this study, it is evident that the choice of strain to be used as biofertilizer should not only be based on nitrogenase activity but also on the ability to produce phytohormones that prevent mycelial growth of rhizosphere pathogens. Of all strains isolated, strain NUL3-2, MAF7-1 and MAF7-3 are potential strains that can be used for further studies as biofertilizer and PGPR.

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