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Screening of Diazotrophs as Plant Growth Promoters and Their Effect in the Development of Maize Seed

Phokoane Connie Monokoane[1], Sissay Bekele Mekbib[2] & Li Haiying[3]

[1] Department of Range Resource Management, Ministry of Forest and Land Reclamation, Maseru, Lesotho

[2] Department of Biology, Faculty of Science and Technology, National University of Lesotho, Lesotho

[3] School of Life Sciences, Heilongjiang University, China

Correspondence: Sissay Bekele Mekbib, Department of Biology, Faculty of Science and Technology, National University of Lesotho, P. O. Roma 180, Lesotho. E.-mail: sbmekbib@nul.ls; sbmekbib7@gmail.com

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Abstract

Free-living diazotrophs play an important role as plant growth promoters by supplementing nitrogen to deficient soils. In this study, six diazotrophic isolates were selected from 36 previously screened diazotrophic isolates for their mode of action to promote plant growth. A nitrogen free medium was used to screen nitrogen fixing diazotrophs. All strains have exhibited some activity of indole-3-acetic acid production. Strain MAF7-1 shown the highest (0.448 mg ml^{-1}), while strain NUL3-2 exhibited the least (0.032 mg ml^{-1}) production of IAA. Though all isolates were unable to solubilize inorganic phosphate, were able to inhibit the growth of *Fusarium oxysporum* that causes wilting to plants. There was no significant difference in maize seed germination vigour and rate. However, MAF7-1 had better germination rate and seed vigour (27.5%) when compared to the control (16%). Except strain NUL3-2, all showed significant increase of root length and dry weight of maize seedlings. Therefore, the choice of strain to be used for production of biofertilizer should not only be based on the nitrogenase activity, but also on ability to produce phytohormones. Strain NUL3-2, MAF7-1 and MAF7-3 are potential strains that can be used in the production of biofertilizer. Further study of these strains in depth as plant growth agents and biofertilizer is commendable for future application.

Keywords: *Azosprillium*, *Azotobacter*, biofertilizer, nitrogen fixing microorganisms, plant growth promoter organisms, *Rhizobium*

1. Introduction

Nitrogen (N), being a constituent of amino acids and nucleic acids, it remain as growth limiting nutrient to all living things. Most of the soils in the whole world lack nitrogen (Postaga, 1998; Hansch & Mendel, 2009). 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 & Kidder, 2003). Therefore, nitrogen reserves of agricultural soils have to be replenished periodically in order to maintain an adequate or non-growth limiting level for crop production (Hubbell & Kidder, 2003).

In order to restore the soil nitrogen, addition of nitrogen in the form of commercial inorganic fertilizers or the use of biological nitrogen fixation (BNF) is important. The application of BNF offers an economically attractive and ecologically sound means of reducing external nitrogen input and improving the quality and quantity of internal sources (Hollister et al., 2013). Rhizosphere microbial communities besides maintaining soil fertility, they also serve as Plant Growth Promoting Rhizobacteria (PGPR) to prevent the deleterious effects of pathogenic organisms by the production of phytohormones through phosphate solubilization, nitrogen fixation (Glick et al., 1999; Lugtenberg & Kamilova, 2007; Beneduzi et al., 2012; Ochoa-Velasco et al., 2016).

In Lesotho, more than 85% of the soil is acidic and deficient in assimilable inorganic phosphate (H_2PO_4-) (Mosenene, 1999). According to FAO/WFP (2004), food production had been decreasing with time due to poor soil quality. In order to improve crop yield, most farmers in Lesotho are using chemical nitrogen and phosphorus fertilizers (Mosenene, 1999). The use of chemical fertilizers is not affordable to these resource poor farmers and intensive use of the synthetic nitrogen fertilizers also affect the environment with nitrogen metabolites (Karmer

et al., 2006). Therefore, the selection and *in vitro* evaluation of diazotrophic isolates was conducted to study their PGPR character, phosphate solubilizing, maize seed growth promotion and their potential in agriculture as plant growth agents and biofertilizer.

2. Methods

2.1 Soil Sample Collection

Six diazotrophic isolates originally were selected from 36 nitrogen fixing isolates from six different regions of Lesotho soils: Muela, Mahobong, Hlotse, Peka, Ha-Mafefooane and the National University of Lesotho campus (Monokoane et al., 2016) were used. Strains were kept in nutrient agar slants at 4 °C until the following trials.

2.2 Nitrogenase Activity Assay

Nitrogenase activity of the selected isolates was measured by acetylene reduction assay (Dilworth, 1966). The vial were filled with 10 mL of the isolates 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 was left to stand for 24-48 h at 28-30 °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.

$$N = (hx \times C \times V)/(hs \times 24.\ 9 \times t) \quad (1)$$

Where, N = the concentration of C_2H_4 (nmol ml^{-1} h^{-1}); hx = the peak value of the sample: C = the concentration of standard C_2H_4 (nmol ml^{-1} h^{-1}); V = the volume of the vial; hs = the 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.

2.3 Screening for PGPR Traits

2.3.1 Indole Acetic Acid (IAA) Production

The purified isolates with nitrogen fixing ability were tested according to Bric et al. (1991) for their ability to produce IAA. The tests were done by preparing liquid Nitrogen Free medium with and without tryptophan (500 µg/ml). A 100 ml of aliquot medium were inoculated with standard concentration (10^8 cfu ml^{-1}) of potential strains and incubated at 25 °C for 7 days on the shaker at 120 rpm. Subsequently, two millilitres of the broth culture was centrifuged at 10,000 rmp for 15 min and one millilitre of the supernatant was mixed with 100µl of 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 until the formation of pink colour. The absorbance was measured spectrophotometrically at 530 nm and data was recorded.

2.3.2 Phosphate Solubilization

The phosphate solubilising activity of the isolates were tested on a plate using a modified Pikovskaya's culture medium (Subba, 1999). Inoculated plates were incubated for 3 days at 25 °C and the experiment was done in triplicate.

2.3.3 Antagonistic Activity

In vitro screening for antagonistic activity was done using the dual culture technique. The isolates were challenged against *Fusarium oxysporum*, a fungus that causes fusarium wilt. The nitrogen free agar plates were spread with the isolates and the block of *F. oxysporum* were placed at the center of the plate. The experiment was done in triplicates. The control plates were not inoculated with the isolates and plates were then kept for seven days at 25 °C for growth. The presence or absence of fungal growth was visually inspected and if growth appeared, data was recorded by measuring the mycelial radial growth.

2.4 In vitro Evaluation of Plant Growth Activity

The seeds were surface sterilized with 1% NaOCl for 30 min, followed by 90% ethanol for 2 min and rinsed 3-4 times with sterilized water. The seeds were inoculated with standard concentration (10^8 cfu ml^{-1}) of isolates and left in contact with the inoculants for 2 hrs. Sterilized water was used as a control. The seeds were then spread evenly in Petri-dishes (50 seeds per 17 cm dish) containing sterile, pre-wetted cotton and placed in the dark for 7 days where the temperature was 24-28 °C. The standard application of the two species: *Rhizobium fredii* (15067) and *Azotobacter chroococcum* (10006) were used as control to compare with that of the isolates. By the end of the 2nd week, the seedlings were dried in order to determine dry weight. Seed germination vigour and germination rate was calculated using the following formula (Jalink & van der Schoor, 1999).

$$Germination\ (\%) = \left(\frac{Number\ of\ seeds\ which\ germinated\ seeds\ at\ the\ beginning\ of\ germination\ period}{Total\ number\ of\ seeds} \right) \times 100 \quad (2)$$

$$Germination\ Index\ (GI) = \Sigma(G_t/D_t) \quad (3)$$

Where, G_t is number of germinated seed at the D_t day after planting and D_t is the total number of days from the day of planting.

$$Vigor\ Index\ (VI) = GI \times S \quad (4)$$

Where, S is the dry weight of the seedlings.

2.5 Statistical Analysis

SPSS software was used to analyze the data. Covariate analysis was used to see the influence of the isolates on the germination of maize seeds. Duncan's multiple range test was applied to estimate the differences between the means at a level $P < 0.05$.

3. Results

3.1 Isolation of Diazotrophs

Six free-living strains of diazotrophic isolates selected from 36 previously screened nitrogen fixing isolates of slightly acidic (pH 5.32) to slightly alkaline (pH 7.2) soils of Lesotho are depicted in Table 1. The strains are grouped under the three genera: *Azosprillum*, *Azotobacter* and *Bacillus* (Table 1).

Table 1. Diazotrophic strains screened for their plant growth promoting potential from slightly acidic soils of Lesotho

Place	Soil Sample	pH	No. of Isolated Strains	Strains Code	Genera
Peka	PEK1	6.43	4	PEK1-1	*Azosprillum*
Peka	PEK5	6.32	1	PEK5-1	*Bacillus*
NUL	NUL3	6.93	2	NUL3-2	*Azospillum*
NUL	NUL18	6.60	1	NUL18-1	*Azotobacter*
Mafefooane	MAF7	6.79	3	MAF7-1	*Azotobacter*
				MAF7-3	*Azosprillum*

Note. The codes designated to strains 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 PEK1-1, shows that the isolate was obtained from the first soil sample from Peka, and it was the first isolate.

3.2 Nitrogenase Activity

All the six screened diazotrphic strains showed nitrogenase activity with the concentration range of C_2H_4 between 97.01-190.20 nmol ml^{-1} h^{-1} (Figure 1). Strain NUL3-2 and PEK5-1 exhibited the highest 190.2 nmol ml^{-1} h^{-1} and, the lowest (97.012 nmol ml^{-1} h^{-1}) nitrogenase activity (Figure 1).

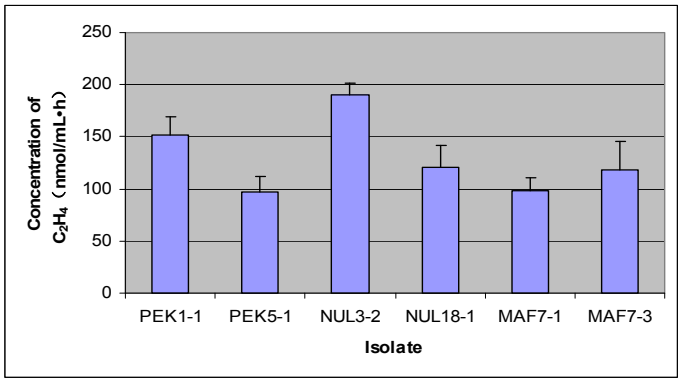

Figure 1. Nitrogenase activity of selected diazotroph isolates

3.3 Screening for PGPR Traits

3.3.1 IAA Production

All selected diazotrophic strains showed some activity of IAA production with the range between 0.04 mg/ml-0.45 mg/ml (Figure 2). The two strains: (MAF7-1 and MAF7-3), produced > 0.4 mg/ml of IAA (Figure 2).

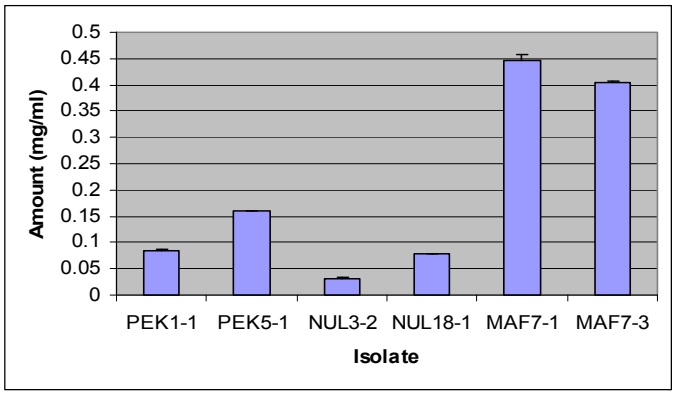

Figure 2. IAA production ability of diazotroph isolates

3.3.2 Phosphate Solubilisation

None of the isolates were shown positive results for solubilisation of inorganic phosphates.

3.3.3 *In vitro* Antagonistic Activity

All strains of the isolated diazotrophs showed some activity of inhibition of growth of *Fusarium oxysporum*. Strain PEK1-1 exhibited significant growth inhibition activity when challenged against *F. oxysporum* (Figure 3).

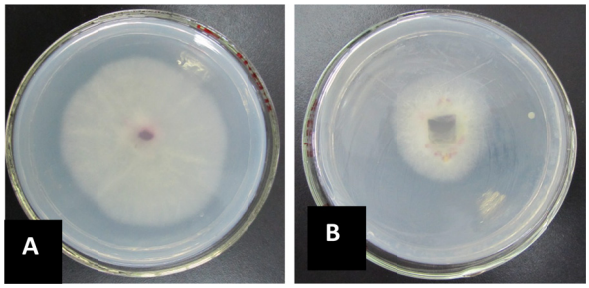

Figure 3. Antagonistic activity of PEK1-1 against *F. oxysporum*

Note. A: control, B: PEK1-1 against *F. oxysporum*.

3.4 In vitro Diazotrophs Activity on Maize Plant Growth

3.4.1 Influence on Plant Biomass

Three strains of the diazotrophic isolates: PEK1-1, MAF7-1 and MAF7-3 showed significant increase ($p < 0.05$) in roots development and biomass increase compared to the standard (Figure 4).

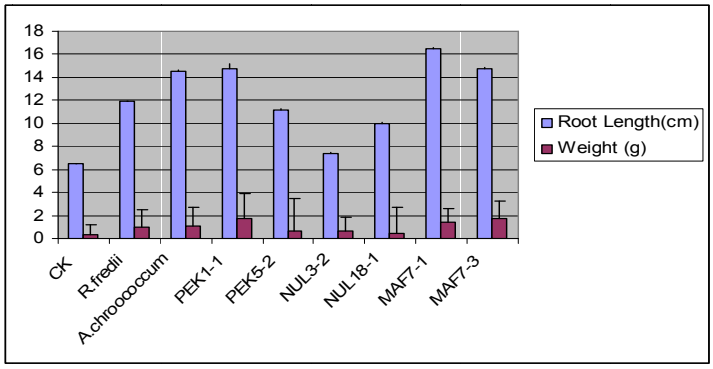

Figure 4. Influence of diazotrophs on the root length and dry weight of maize seedlings

3.4.2 Efficacy on Maize Seed Germination and Indexing

All strains showed positive effect on maize seeds germination except NUL3-2 (Figure 5). On the other hand, strain MAF7-1 exhibited the highest activity on maize seed vigour and germination (Figure 5). Treatment application with *A. chroococcum*, PEK1-1 and MAF7-1 had shown significant difference at $p < 0.05$ for germination index (Table 2). Seeds treated with strain MAF7-1 shown the highest while seeds treated with NUL3-2 shown the lowest germination index (Table 2). Except strain PEK1-1 that sowed the highest performance, all strains exhibited positive effect on seed vigour indexing (Table 2).

Table 2. Efficacy of diazotroph strains on maize seed germination rate and indexing by the end of the 7th day

Isolate	Germination energy (%)	Germination rate (%)	Germination index	Vigor index
Ck	16	83	18.2	4.7
Rhizobium fredii	16	89.5	19.4	20[a]
Azotobacter chroococcum	28	92.5	21.8[a]	24[a]
PEK1-1	28	87	20.9[a]	32.5[Aa]
PEK5-1	24	81.5	19.6	14.5[a]
NUL3-2	16.5	78.5	16.2	11.2
NUL18-1	24.5	76	20.3	8.3
MAF7-1	27.5	75	22.3[a]	30.2[a]
MAF7-3	24.5	87	20.4	29.9[a]

Note. Letters represent significant difference when compared with the control at $P < 0.05$ significance level.

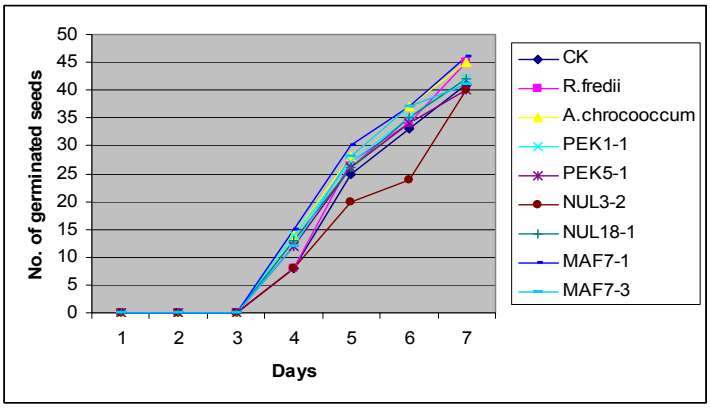

Figure 5. Effect of diazotrophs on maize seed germination rate

4. Discussion

In this study, six strains of diazotrophic isolates, three of which: PEK1-1, NUL3-2, and MAF7-3 belong to the genus *Azospirillum*; two strains: NUL18-1 and MAF7-1 that belong to genus *Azotobacter* and strain PEK5-1 that belongs to the genus *Bacillus* were evaluated for their maize seed vigour and plant growth activity. All the isolated strains have shown some ability of producing Indole Acetic Acid; of which, the *Azotobacter* strain, MAF7-1 found producing the largest amount followed by the *Azospirillum* strain, MAF7-3. Reports had shown that many plant-associated bacteria have the ability to produce the plant growth regulator indole-3-acetic acid (IAA) that may play the most important role in plant growth promotion (Fogaca & Fett-Neto, 2005; de-Bashan et al., 2008). Indole acetic acid secreted by a bacterium may promote root growth directly by stimulating plant cell elongation or cell division or indirectly by influencing bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Lambrecht et al., 2000; Bashan & de-Bashan, 2010).

The presence of high number of bacteria in the rhizosphere is undoubtedly also important, since they may convert organic and inorganic substances into available plant nutrients. Plant growth is frequently limited by unavailability of soluble phosphates in the soil, which are considered one of the most important growth-limiting environmental factors (Raghothama, 1999). The low solubility of common phosphates, such as $Ca_3(PO_4)_2$, hydroxyapatite and aluminum phosphate causes low phosphate availability. Some bacteria are able to secrete organic acids and enzymes that can act on insoluble phosphates and convert it into soluble forms, thus, providing phosphorus to the plants (Gadagi et al., 2004). However, in this study none of the isolated strains was able to solubilise phosphates.

Plant growth promoting rhizobacteria and bacterial endophytes play a vital role in the management of various fungal diseases (Ochoa-Velasco et al., 2016). In this study, an *in vitro* antagonism activity test of *Azospirillum* strain against *F. oxysporum* has shown significant suppression effect against the mycelial growth of the pathogen.

Similar results have been reported by Beneduzi et al. (2012) against soil borne pathogens. Rhizobacteria belonging to the genera *Pseudomonas* and *Bacillus* are well known for their antagonistic effects and their ability to trigger induced systemic resistance against soil borne pathogens (Audenaert et al., 2002; Sharma & Johri, 2003; Beneduzi et al., 2012; Babu et al., 2015).

It has been assumed that inoculation with diazotrophic bacteria like *Rhizobium*, *Azotobacter* and *Azospirillum* enhanced the plant growth as a result of their ability to fix nitrogen. However, despite of extensive research efforts, only rhizobia have been shown to increase yields from nitrogen fixation. Therefore it can be assumed that growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other plant growth promoting (PGP) activities (Frankenberger & Arshad, 1995; Yang et al., 2009; Andress et al., 2015). This study also revealed that there is no direct link between level of nitrogenase activity with promotion of plant growth, as the strain which had highest level of nitrogenase activity did not have highest influence on plant growth (Khalid et al., 2004). However, the strains that produced the highest level of IAA had great influence on germination of maize seeds, which supports the results reviled by Frankenberger and Arshad (1995).

Successful and uniform germination is also important in agriculture. The quality of crops depends on optimal germination and plant development. There was no significant difference in germination vigour and germination rate between the control and diazotrophs. However, some of the strains had shown influence on germination and vigour index. Seeds inoculated with MAF7-1 exhibited the highest germination index, while NUL3-2 exhibited the lowest. The germination index has been shown to be a very sensitive index of phytotoxicity (Rodriguez & Fraga, 1999). The highest vigour index was obtained from PEK1-1 followed by MAF7-1 and MAF7-3, respectively. High vigour seeds have distinct growth ascendancy and production potential, which can improve field emergence, resist environmental stress (Yang et al., 2009), and increase crop yields (Rodriguez & Fraga, 1999).

Plant growth promotion and antagonistic activity of *Rhizobium*, *Azotobacter* and *Azospirillum* might be useful in formulating new inoculants with combinations of different mechanisms of action, leading to a more efficient use for biocontrol strategies to improve cropping systems.

5. Conclusion

In this study, all the strains identified in the three genera have shown the ability to produce IAA and prevent mycelial growth of *F. oxysporum*. However, they were unable to solubilize inorganic phosphate. In this study, it was evident that there was no significant direct correlation between nitrogenase activity and plant growth promoting activity. Therefore, the choice of strains to be used as biofertilizer should not only be based on the nitrogenase activity, but also on the ability of producing phytohormones. Strain NUL3-2, MAF7-1 and MAF7-3 are potential strains that can be used for further studies in this area of biotechnology.

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