ISOLATION AND CHARACTERIZATION OF BACTERIA WITH BIOFERTILIZER POTENTIAL

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Abstract: Biofertilizer is an environmental friendly alternative to chemical fertilizers to increase soil fertility. It is also used in crop production in sustainable farming, because the long term use of chemical fertilizers destroy the friability of the soil. Soil samples were collected from different agricultural farms in Ibadan Oyo State. The samples were collected with clean ziplock polythene bags and transported to the laboratory on ice pack. Total bacteria counts, plant growth promoting abilities of the isolates such as nitrogen fixation, phosphate and potassium solubilization were determined using nutrient agar, glucose nitrogen free media, Pikovskaya and Aleksandrouv media, respectively. The highest total bacteria count on nutrient agar was recorded for sample H (16.8) (Nihort farm) whereas the lowest count was recorded for sample C (6.90) (Southern farm). The isolated organisms used for the inoculation were *Micrococcus* sp, *Pseudomonas* sp, *P. fluorescence*, *Bacillus* sp, *Alcaligens* sp and *Proteus* sp. It has been observed that the isolates have the ability to fix nitrogen, solubilize phosphate and potassium. It was also observed that the control had the lowest percentage germination. In conclusion, it was observed that the isolates were capable of improving the plant growth in terms of leaf length, root length and percentage germination.

Keywords: Biofertilizers, bacteria, chemical fertilizers, germination, microorganisms, soil

Introduction

Soil microorganisms have been used in crop production for many years (Hayat *et al*., 2010). Microbes in the soil are directly tied to nutrient recycling especially carbon, nitrogen, phosphorus and sulfur. Bacteria are the major class of microorganisms that keep soils healthy and productive. Here are some main functions of bacteria to the soil. They supply nutrients to the crops, it helps in plant growth, it controls the activities of plant pathogens and it improves the soil structure. Bacteria can be used for bioremediation in polluted soils Hayat *et al*. (2010).

Bacteria are tiny and one-celled organisms. A ton of microscopic bacteria may be active in each acre of land. Bacteria are similar to clay soil particles in size (Hoorman, 2016). Bacteria grow and live in thin water films around soil particles and near roots in an area called the rhizosphere. Because bacteria is small in size, this enables them to grow and adapt more rapidly in most soils. Bacteria population can multiply easily in double within 30minutes. Flourishing microbial populations increase soil productivity and crop yields over time. The oxygen levels of the soils determines the activities of soil bacteria. Most bacteria in the soils prefer oxygenated soils and make use of the oxygen to decompose most carbon compound in the soil. Example of this bacteria include Aerobacter, Actinomycetes and Streptomyces which allow the soils to remain in its nature form (Lowenfels and Lewis, 2006).

The development and use of microbial-based fertilizers has recently gained significance due to the recognition of the deleterious effects on the environment generated by the excessive and improper application of chemical fertilizers (Maurya *et al*., 2014). This was a result of the improved knowledge about the relationships occurring in the rhizosphere, between the plant and all soil microorganisms, as well as due to the immense efforts in isolating and selecting microbial strains showing plant growth promoting capabilities (Parmar and Sindhu, 2013).

Biofertilizers have been identified as an alternative to chemical fertilizers to increase soil fertility and crop production in sustainable farming. It have emerged as an important component of the integrated nutrient supply system and hold a great promise to improve crop yields through environmentally better nutrient supplies (Wu *et al*., 2005). Biofertilizers improve root proliferation due to the release of growth promoting hormones. Microorganism converts complex nutrients into simple nutrients for the availability of the plants. Biofertilizers are products of one or more species of microorganisms which have the ability to mobilize nutritionally important elements from non useable to useable form through biological processes such as nitrogen fixation, phosphate solubilization, excretion of plant growth promoting substances and biodegradation in soil. Biofertilizers are living microbial inoculants of bacteria, algae, fungi alone or in combination. The role of biofertilizers in agriculture assumes special significance, particularly in the present contex of increased cost of chemical fertilizers and their harzadous effects on soil health (Kumar *et al*., 2017). Some of the microorganisms used as biofertilizers include bacteria (*Rhizobium*, *Bradyrhizobium*, *Azospirillum*, *Azobacter*, *Bacillus*, and *Pseudomonas* species) and fungal species such as mycorrhizal fungi, *Penecillium*, *Chaetomium* and *Trichoderma* (Kaechai and Hyde, 2009).

Application of bio-fertilizers and biosludge to arable soil influenced physical and chemical properties of soil but as well as structure and function of soil microbial community (Cercioglu, 2014). This group of bacteria are of great importance in cycling nutrients such as carbon (C), nitrogen (N), phosphorus (P), and sulphur (S). Not only do they control the forms of these elements (for example, specialized soil bacteria convert ammonium N (NH_4^+) to nitrate (NO_3^-) they can regulate the quantities of Nitrogen (N) available to plants. Beside their effects on the availability of nutrients, the bacterial soil prevents the uptake of several harmful ions. The use of living bacteria (biofertilizer) accelerates mineralization of organic residues in soil, therefore makes the nutrients more available to crops. However, the aim of this study was to evaluate the effect of phosphate solubilizing, nitrogen fixing and potassium solubilizing ability bacteria on seed germination of plants.

Materials and Methods

Experimental site

The research work was carried out in the Institute of Agricultural Research and Training, Moor-Plantation, Ibadan Oyo State.

Sample collection

Soil samples were collected from different agricultural farms in Ibadan Oyo State. The farms are listed as follows; Southern farm (IAR&T), Reck animal production technology farm (IAR&T), Reck Quarters farm (IAR&T), Pathology farm (IAR&T), Forestry Farm and Nihort Farm. The samples were

collected with sterile polythene bag and transported to the laboratory for analysis on ice pack.

Isolation of microorganisms from the soil samples

Five (5) grams of the soil samples were suspended in 250 ml Erlenmeyer flasks and incubated in an orbital shaker at 30^0C and 120 rpm for 5 days after which 1 ml of the culture was serially diluted and plated on Pikovskaya, Aleksandrov and Jenson media and incubated for 72 h. The bacterial counts on the respective media were recorded after 72 h of incubation (Tan *et al*., 2014). Morphological distinct colonies were sub cultured repeatedly until the cultures were pure. The isolates were stored on agar slant for further studies.

Screening for phosphorus and potassium solubilization and nitrogen fixing abilities

The isolates were spot inoculated at the centre of prepared Pikovskaya and Aleksandrov plates and incubated for 72 h at 30^0C. The formation of clear zones around the colonies indicates phosphate and potassium solubilization, respectively (Tan *et al*., 2014). The solubilization ability of the isolates were determined by measuring the solubilization index while the nitrogen fixing ability by the isolates were assessed using glucose nitrogen free mineral medium (GNFM). The ability of the isolates to fix N_2 was observed by the change in colour of the medium after the incubation period to blue. The medium without the inoculants was used as control (Bashir *et al*., 2013)

Identification of the isolates

The selected plant growth promoting isolates were identified using morphological and biochemical characteristics (Holt *et al*., 1994).

Plant inoculation test

The isolates with best plant growth promoting abilities were used for plant inoculation test. Maize seeds were surface sterilized with 0.1% aqueous mercuric chloride, the seeds were placed in a suspension of bacterial cell diluted to 0.5 Mcfarlands standard for 30 min while the control was placed in a sterile distilled water. Thereafter they were planted in a low nutrient soil (sea sand) moistened with sterile distilled water and were grown in a controlled chamber for 7 days. The length of the root and shoot as well as shoot dry weight were measured after 7 days and compared with the control. The seed germination rate was determined after 3, 5 and 7 days of planting (Akintokun *et al*, 2019).

Results and Discussion

The highest total bacterial count on nutrient agar was recorded from National Horticultural Research Institute farm Ibadan (NIHORT Farm (H)) whereas the lowest count was recorded from Southern farm Institute of Agricultural Research and Training moor plantation, Ibadan (Southern Farm (C)). In Pikovskaya media, it was observed that sample C which was Southern farm was significantly higher in the isolates while sample B which is from Reck Animal Production Technology Moor-Plantation Ibadan was significantly lower in values. For Jenson isolates, the values ranged from 5.8 to 8.2x10^6 Cfu/ml and there was no significant difference between number of isolates from F (IARandT Farm) and G (Forestry Farm). For Aleksandrov medium, the values of the isolates ranges from 3.2 to 8.1 x 10^6 Cfu/ml (Table 1).

Table 1: Bacteria count from different isolation media

Location	(×10^6Cfu/ml)			
	Alex	Jenson	Pikovskaya	NA
A	3.2	6.0	7.2	15.0
B	8.1	8.2	3.3	13.2
C	7.3	7.0	7.4	6.90
D	6.3	5.9	6.9	16.2
E	6.8	6.8	6.3	10.6
F	6.7	6.9	7.1	12.0
G	6.5	6.9	6.9	15.0
H	4.8	5.8	7.0	16.8

A-Southern Farm (FCA) **B**-Reck Animal Production Technology **C**-Southern Farm (IAR&T) **D**-Reck Quarters **E**- (IARandT) Farm Adjacent Pathology **F**- IARandT Farm **G**-Forestry Farm **H**- Nihort Farm.

Table 2: Morphological appearance of the isolates with their reaction to gram stain

Gram stain	Morphology	Probable Organisms
Gram positive spherical shape	Bright yellow colonies	*Micrococcus* species
Gram-negative Rod shape	Blue- green colonies	*Pseudomonas* species
Gram - negative Rod shape	Yellow-green colonies	*Pseudomonas* fluorescence
Gram positive Rod shape	Cream colonies	*Bacillus* species
Gram negative Rod shape	Golden yellow colonies	*Alcaligenes* species
Gram negative Rod shape	Yellowish brown colonies	*Proteus* species

Table 2 shows morphological appearance of all the isolates from all the media used. The organisms isolated were *Micrococcus* species, *Pseudomonas* species, *Bacillus* species, *Alcaligenes* species *and Proteus* species. Morphologically the *Micrococcus* species showed bright yellow colonies on the media and gram positive spherical shape. The *Pseudomonas* species are gram negative rod shape with blue-green colonies. *Bacillus* species were gram positive rod shape with cream colonies. *Alcaligenes* species were gram negative rod shape with golden yellow colonies and *Proteus* species were gram negative rod shape with yellowish brown colonies. Biochemical characteristics of the isolates varied from one isolate to the other. All the isolates were negative to indole test, all were positive to catalase test except B2 (*P. fluorescence*). All the isolates were positive to oxidase test except B1 (*Proteus* specie). Also, some isolates were positive to urease, starch hydrolysis, citrate and voges-proskauer whereas some were negative to them. Some isolates produced gas whereas only some isolates from D3 produced acid and gas Table 3.

Table 3: Biochemical characteristics of the bacterial isolates

Biochemical Test	Isolate codes							
	A2	D2	B2	D3	C1	A3	B1	E1
G/stain	+	-	-	+	-	+	-	+
Cat	+	+	-	+	+	+	+	+
Ox	+	+	+	+	+	+	-	+
Ind	-	-	-	-	-	-	-	-
Glu	+A	+	+	+AG	-	+A	+G	+
Man	+A	+	+	+AG	-	+A	-	+
Lac	+A	+A	+A	+AG	+	+A	-	+
Fru	+A	+A	+A	+AG	-	+A	-	+
Ure	+	+	+	+	-	+	+	+
S/hyd	-	+	+	+	+	-	+	+
Cit	-	+	+	-	+	-	+	-
MR	-	-	-	-	-	-	+	-
VP	-	+	+	-	-	-	-	+

Probable bacteria: A2- *Micrococcus* sp., **D2**-*Pseudomonas* sp. **B2**-*P. fluorescence*, **D3**- *Bacillus sp*., **C1**-*Alcaligenes* sp., **A3**- *Micrococcus* sp., **B1** -*Proteus sp*,**E1**-*Bacillus* sp; cat-catalase, Ox-oxidase, Ind-indole test,Glu-glucose, Man-manitol, Lac-lactose, Fru-fructose, S/hyd-starch hydrolysis, Cit-citrate utilization, MR-methylred, A-acid production, A/G-acid and gas production, VP-voges-proskauer.

Table 4: Plant growth promoting abilities of the isolates

Isolates	P solubilizer	K solubilizer	Nitrogen Fixing ability
A1	+	-	+
A2	+	+	+
A3	+	+	+
A4	+	-	-
B1	+	+	+
B2	+	+	+
B3	+	-	-
B4	+	+	-
C1	+	+	+
C2	+	-	-
C3	+	+	-
C4	+	-	-
D1	-	+	-
D2	+	-	+
D3	+	+	+
D4	+	-	-
E1	+	+	+

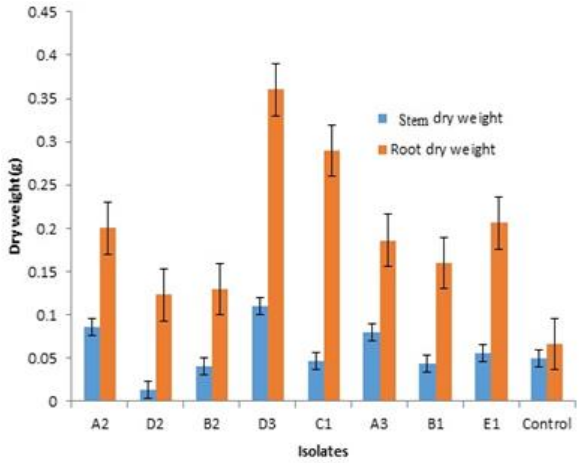

Fig. 1: Effect of the isolates on the stem and root dry weight of maize

Table 4 shows that the isolates have the ability to fix nitrogen, solubilize phosphate and potassium. The D3 (*Bacillus* species) has the highest root dry weight and has the highest stem dry weight while the control has the lowest root dry weight and D (*Pseudomonas* specie) has the lowest stem dry weight Fig. 1. This report shows that all the isolates were phosphate solubilizer except the isolate code D1 which is negative. Also some isolates were potassium solubilizer while some were not and in all the isolates about 55% had nitrogen fixing ability while 45% among the isolates were not. Cheng and Fanyu (2014) reported that potassium (K) is essential to plant growth and development; it helps in the utilization of Nitrogen (N), and synthesis of protein and sugar. In plants, K deficiency causes yellowing of the leaf edges and can also lead to slow growth and incomplete root development. Previous studies have shown that potassium solubilizing bacteria (KSB) can promote plant growth. The study has indicated that inocula can enhance plant growth and increase plant K content. Potassium solubilizing microorganisms play vital role in making available insoluble forms of potassium by mineralization (Shanware *et al*., 2014). Satya *et al*. (2017) reported about phosphate solubilizing bacteria (PSB) that they are ubiquitous and varies in shape and they are present in different types of soils. It was further reported that their population in soil depends on the chemical and physical properties of the soil, also the organic matter and phosphorus content of the soil. The breaking down of insoluble phosphate into soluble form is done by some microbes present in the soil (Prajapati and Modi, 2012). Microorganisms with phosphate solubilizing potential increase the availability of soluble phosphate and enhance the plant growth by improving biological nitrogen fixation. In this study, the isolates that were used as biofertilizers are *Micrococcus* sp, *Pseudomonas* sp, *P. fluorescence*, *Bacillus* sp, *Alcaligenes* sp, *and Proteus* sp. this correlates with the study of Sharma *et al*. (2011) deduced that there are groups of bacteria species that have beneficial effect on the plant growth and can be used as biofertilizers and some of these organisms are *Alcaligenes*, *Bacillus*, *Enterobacter* and *Pseudomonas*. Nitrogen enters ecosystems predominantly through bacterial fixation. Biological nitrogen fixing microorganisms also improve the soil microbiological activities. Legume nitrogen fixation recovers the losses of nitrogen from the soil, hence soil become more productive and fertile (Bano and Iqbal, 2016).
Figure 2 shows the effect of the isolates on the leaf length and the root length. It was observed that seeds treated with isolates A2 and E1 had the highest leaf length whereas control had the lowest value for root length.

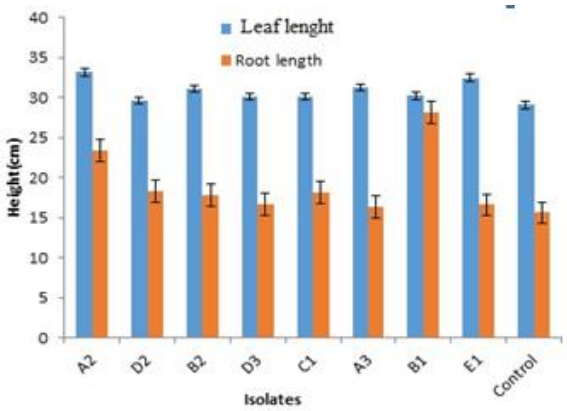

Fig. 2: Effects of the Isolates on the leaf length and root length

Table 5: Effects of the isolates on maize germination

Isolate	% Germination			
	3rd day	5th day	7thday	No. of leaves
A2	98.65±2.81^{a}	98.65±2.81^{a}	98.65±2.81^{a}	3.00±0.00^{b}
D2	100±0.00^{a}	100±0.00^{a}	100±0.00^{a}	2.33±0.56^{b}
B2	73.3±3.055^{b}	78.30±2.08^{b}	78.3±2.08^{b}	2.66±0.53^{ab}
D3	83.3±2.51^{ab}	83.30±2.52^{b}	83.3±2.51^{b}	2.33±0.53^{a}
C1	80.65±2.08^{c}	80.65±2.08^{c}	80.65±2.08^{c}	3.00±0.00^{b}
A3	98.65±2.81^{a}	98.65±2.81^{a}	98.65±2.81^{a}	3.00±0.00^{b}
B1	91.65±2.81^{a}	98.65±2.81^{a}	98.65±2.81^{a}	3.00±0.00^{b}
E1	99.60±2.81^{a}	99.60±2.81^{a}	99.60±2.81^{a}	3.00±0.00^{b}
Control	73.3±0.57^{b}	73.3±0.58^{d}	73.3±0.57^{d}	2.00±0.00^{a}

Means with the same superscript with the column are not significantly different (P≤0.05)

It was observed that all the seeds planted germinated. Table 5 showed that control has the lowest percentage germination in 3rd, 5th and 7th days of germination except isolate B2 for the third day had the same value with the control which indicates that all the isolates were able to increase soil fertility. Table 5 showed the effect of isolates on maize germination. On the third day of planting, it was observed that there was no significant difference among isolates A2, D2, A3, B1 and E1. They have the highest range of percentage germination. On the fifth day of germination, the values ranges from (73.3 – 100%). On the seventh day of germination, the values range

from 73.3 - 100%. Fernandes and Bhalerao (2015) reported that biofertilizers are commonly called microbial inoculants which are capable of making use of non usable nutritional elements in the soil to usable form by the crop plant through their biological processes. Further report said that biofertilizers fix the atmospheric nitrogen in the available form for plants. Biofertilizers are low cost, it is renewable sources of plant nutrients which supplement chemical fertilizers. It helps to reduce the use of chemical fertilizers for sustainable agriculture. Fernandes and Bhalerao (2015) further reported that when *Vigna radiata* plants were treated with biofertilizer *Rhizobium japonicum*, it showed excellent result compared to control plants. The work further reported that all other plants treated with biofertilizers showed significant improvement in the growth like the number of leaves, length of leaves, breadth of leaves, length of plants, shoot length and root length. This is in accordance with this present study which proves that *Pseudomonas* sp, *Bacillus* sp and *Micrococcus* sp showed the highest percentage germination at 3rd, 5th and 7th day after planting. The least percentage of germination was observed in the control (without microbial inoculant) and the seed inoculated with isolate B2 which had the same value with the control only on the 3rd day after planting. The report of Iwuagwu *et al.* (2013) described biofertilizers as substances which contain living organisms and when applied to seed, plant surface, or soil colonize the plant and promote its growth by increasing the nutrient availability. The work further discloses that the application of biofertilizer alone increased the growth parameters of maize seedlings in terms of plant height, stem base diameter as well as fresh and dry weight of plants, this agrees with this present study that microorganisms used as biofertilizers like *Bacillus* sp (D3) had the highest root dry weight while the control had the lowest root dry weight, it was also observed that *Micrococcus* sp and *Bacillus* sp (A2 and E1) were significantly higher in leaf length likewise the root length of A2 was also significantly higher among others, however control was significantly lower in root length among the rest. The effect of the isolates on maize germination recorded that on 3rd, 5th and 7th day after planting, they all have improved percentage germination compared to the control which was lower in percentage germination value.

Conclusion and Recommendation

In conclusion, It was observed that the isolates (biofertilizers) were capable of improving the plant growth in terms of leaf length, root length and percentage germination. The isolates were able to solubilize phosphate, potassium and fixed nitrogen. Since biofertilizers are capable of improving the soil fertility, it is now recommended that the use of biofertilizers should be encouraged in planting of crops. There should be better awareness among all the stake holders and farmers on the need to utilize the isolates as biofertilizers.

Conflict of Interest

The authors declare that there is no conflict of interest reported in this work.

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