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COMPARATIVE ASSESSMENT OF BIOFERTILIZER POTENTIALS OF FUNGAL AND RHIZOBACTERIAL ISOLATES

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ABSTRACT

Pollution and degradation of soil due to continuous use of chemical fertilizers has led to the screening of microorganisms for use as bio-fertilizer by reason of their cost effectiveness and eco friendliness. This work investigated the ability of microorganisms to enhance crop growth using Zea mays as a case study. Pantoea eucaria, Bacillus wiadmanii and Aspergillus flavus isolated from rhizosphere of maize and identified molecularly were tested. Soil samples were obtained from Institute of Agricultural Research and Training, Moor plantation, Ibadan. Ability of the isolates to produce growth promoting factors were assessed using pikovskaya, Alecksandrouv and glucose nitrogen free media before inoculated into the seed of the maize. Three pots in replicates, each contained 5 kg of soil were seeded. Isolates were applied separately and in combination with two controls. They were allowed to stand for six weeks under green house conditions, agronomic parameters were measured at interval. Result showed ability of the isolates to solubilized Phosphorus, potassium and fix nitrogen. Pantoea eucaria and Aspergillus flavus had P-solubilization index of 1.02 and 0.551 respectively. Indole acetic acid and gibberellic acid production was observed for Bacillus wiadmanii (2.6 mg/l) and Aspergillus flavus (F1) (0.56mg/l) respectively. Height (260 cm) was observed in seeds with NPK fertilizer, (206 cm) for seeds treated with the two bacteria, seeds with Bacillus wiadmannii had the least. The leaf area was not significantly different ($P \leq 0.05$) at different treatment. Plant biomass for NPK fertilizer seeds (118.91g) while the control was 35.32g. The isolates tested can be useful in the formulation of fertilizers, their utilization would help to reduce loss of soil fertility and pollution resulting from continuous use of chemical fertilizers.

Keywords: *Zea mays*, Microorganisms, growth, fertilizers

INTRODUCTION

Chemical fertilizers have been the major substrates used by farmers to boost agricultural products in order to meet up with the rising demand of food in the world especially in Nigeria where poverty is high with increased population growth (Conway, 2012). However, the continuous and indiscriminate use of these fertilizers have led to loss of fertility and degradation with resultant low soil fertility and poor yield (Savci, 2012). According to Sujanya and

Chandra (2011), enormous use of chemical fertilizers in agriculture makes the country self- dependent in providing large amount of food supply but to a great extent damages the environment and causes harmful impact on living beings.

Bio-fertilizers is a modernized form of organic fertilizer in which beneficial organisms have been incorporated (Chen *et al*., 2006). In recent time, it has been recognized as an essential component of the nutrient supply system that has the potential to improve crop yield through environmental friendly nutrient supply (Marianna *et al.*, 2015). Bio-fertilizers have been described as a substance which contain living organisms, and when applied to seeds, plant surfaces or the soil. Colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003). Bio-fertilizers include mainly the nitrogen fixers, phosphate solubilizer, phosphorus solubilizers and mobilizers that are added exclusively or in combination with microorganisms. They add nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus, and stimulating plant growth through the synthesis of growth promoting substances (Faren, 2019). Nitrogen fixing and phosphate solubilizing bacteria are important for plant nutrition as they increase nitrogen and phosphate uptake of the plant and play a significant role as plant growth promoting bacteria in the biofertilization of crops. They also produce essential plant phytohormones such as indole acetic acid (IAA) and gibberellic acid. Some produce antibiotics and enzymes capable of protecting plants from inversion by pest and diseases

Fungi (*Tricoderma*, and yeast), and bacteria (*Bacillus, Pseudomonas, Lactobacillus)* had been used as bio-fertilizers as they encouraged plant yield while rhizobium, blue green algae, and azoilla are crop specific, bioinoculants: *Azotobacter*, *Azospirillum*, phosphorus solubilizing bacteria and vesicular arbuscular mycorrhiza could be regarded as broad-spectrum bio-fertilizers with enhanced accumulation and stimulation of plants nutrients (). The organisms possess the ability to convert insoluble phosphate in soil into soluble forms by secreting organic acids that reduces the soil pH and bring about the dissolution of bound forms of phosphate (Bokhtiar and Sakurai, 2005, Verma *et al.*, 2010). *Bacillus* species however had been reported to have advantage over the other genera because of their ability to produce spore in unfavourable environmental conditions which facilitates the conversion of spore suspensions to power formulations without killing the bacteria (Lalloo *et al*., 2010).

In Nigeria, with increased population and dependent on agricultural produce, farmers are faced with challenges due to decreased productivity of crops as a result of soil fertility problems. According to France and Van Huis, (2016), there is need to improve on the management system, efficiency and sustainable practices of agriculture. In order to prevent more damage to the soil structure by the continual use of chemical based fertilizers. There is need for microbial based fertilizers that have the ability to enhance and restore soil fertility, and also eco-friendly. The study screened for possible microorganisms with potentials that could be used to enrich soil fertility and improve crop production in sustainable farming. The utilization of bacterial and fungal isolates that are cheaper and ecofriendly an alternative to chemical fertilizer will help to reduce the dangers inherent in the continuous use of chemical fertilizer. The side effect posed to soil by the application of chemical fertilizers and the resultant effect on crop yield necessitated the search for alternative fertilizers.

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MATERIALS AND METHODS

Sample collection:

Soil sample attached to the maize roots were collected from Agricultural farm in the I.A.R $ T, Moor plantation, Ibadan.

Isolation of bacteria:

The method of Akintokun *et al*., (2019) was adopted for the isolation of the bacteria. The samples were serially diluted and plated on Aleksandrouv and Pikovskaya media incubated for 36 h. After incubation, the isolates that showed evidence of potassium and phosphorus solubilization were isolated and sub-cultured repeatedly until pure cultures were obtained. The pure cultures were stored on agar slant for further studies.

Screening for Plant Growth Promoting Abilities

Phosphate (P) solubilization test:

Spot-inoculated at the centre of the prepared Pikovskaya plates (Tan *et al.,* 2014) and incubated for 72 h at 30^{o}C. The zones of phosphate solubilization formed around colonies were recorded after 72 h. The solubilization index of the isolates was determined by dividing the total diameter of the halo zone (colony+ halo zone) with the diameter of the colony. Further quantification of P-solubilization was done according to the method of Tan *et al*, (2014).

Potassium (K) solubilization ability:

Bacterial suspension from a day old culture grown on a modified Aleksandrov agar medium was prepared and a loopful was streaked onto the medium and incubated at 30^{o}C for 5 days after which it was observed for clear zone. The ability to solubilize mica powder as a source of insoluble form of potassium was derived by the formation of clear halo zone around the colony. The solubilization index of the isolates was determined by dividing the total diameter of the halo zone (colony+ halo zone) with the diameter of the colony (Tan *et al.,* 2014). Further quantification of the amount of K-solubilized were determined using Atomic absorption spectrophotometer (Akintokun *et al*, 2019)

Indole acetic acid Production (IAA):

Bacterial isolates were inoculated into 100 ml peptone water incubated in an orbital shaker for 24 hrs. One ml of the bacterial culture was transferred into a fresh 100 ml peptone water with the addition of 5 ml of L-tryptophan as a precursor of indole acetic acid (Tan *et al.,*2014). Peptone water without bacterial inoculum served as control. A portion of the bacterial culture was transferred into a sterile tube and centrifuged at 7000 rpm for 7 min. The supernatant was mixed with 2 ml of Salkowsky reagent (2% of 0.5 M FeCl3 in 35% perchloric acid. The solution was allowed to stand for 25 mins and development of a pink colour indicated IAA production. The absorbance values were determined using a spectrophotometer at 535 nm and compared to the standard curve to determine the IAA concentration. The IAA standard curve was prepared using pure IAA at 0, 5, 10, 15, 20, 25, 30 mg/ml of IAA.

Gibberellic acid production:

The isolates were inoculated in Jenson broth and incubated on a shaker for 5 days. The gibberellic acid was extracted from the supernatant using liquid-liquid extraction method (Umi *et al.,* 2014). The broth cultures were centrifuged at 10,000 rpm for 15 min. The supernatants were filtered with separating funnel and pH adjusted to 2.5 using 2N HCl. Ethyl acetate (volume1:10) was added and shaken for 1 min and the liquid phase was transferred to another separating funnel. The extraction procedure was repeated by adding ethyl acetate. To remove impurities, the ethyl acetate fraction was partitioned with 1 M $NaHCO_3$ (Volume1:1). The amount of

gibberellic acid was determined at the ethylacetate phase using spectrophotometer at 254 nm. Uninoculated Jenson medium was used as control.

Molecular identification of the selected isolates

Bacterial genomic DNA was extracted using the protocol stated by Frank *et al.* (2008). Polymerase Chain reaction cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of $MgCl_2$, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 p mol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water and 8µl DNA template. The PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with an initial denaturation, 94°C for 5 min; 30 cycles, of 94°C for 30 secs, 50°C for 60 secs and 72°C for 1 min 30 secs and a final extension at 72°C for 10 mins. And chill at 4°C. The amplified fragments were ethanol purified in order to remove the PCR reagents and sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of Big Dye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis. The DNA sequence was compared with the sequence available in NCBI database using BLAST.

Screen house studies:

The screen house studies were conducted at IART Ibadan Moor plantation, Ibadan. The soil used was weighed, sieved and 5 kg of soil were filled in fifteen perforated plastic pots. All treatments were arranged and laid in Complete Randomized Design (CRD) with three replicates.

Preparation of bacteria Inoculum

The selected isolates were inoculated in 50 ml conical flask containing 25 ml of prepared and sterilized Luria broth and incubated at 30^{o}C in an orbital incubator shaker for 24 h. After incubation, the culture was centrifuged at 2000 rpm for 20 minutes. The cells were harvested and washed with normal saline. In other to ensure equal cell size, the cells were diluted to 0.5 McFarlands Standard to give approximate cell density of 1.5 $X10^8$ CfU/ml.

Seed sterilization and Inoculation

Maize Seeds (Swan 1) were surface-sterilized with 70% ethanol and 2% sodium hypo chloride for 5 mins and then washed with sterile distilled water. The surface sterilized seeds were inoculated with the isolates by soaking in the respective bacterial suspension for 45 minutes

Experimental Design and treatments:

Complete randomized designs with three replicates were used for screen house studies. The following treatments were used:

T_1 - soil+ maize only

T_2 - Soil + maize with *Pseudomonas flourescens*

T_3 - Soil + maize with *Bacillus cereus*

T_4 - Soil +maize with mixed culture of *B. cereus* and *P. flourescens*

T_5 - Soil + N.P.K

T6 -Soil +Maize with *Aspergillus* sp.

Planting of Inoculated Seeds and Determination of growth Parameter

Maize Seeds (Swan1yellow) were inoculated by soaking them in the respective bacterial suspension. Four seed per pot were planted at the screen house and later thinned to 2 plants per pot after two weeks. Growth parameters such as height, girth size and number of leaves were measured 2, 4 and 6 weeks after planting.

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The heights from the soil to the growing tip of the main shoot of the maize were measured using meter rule. A vernier caliper was used to measure the plant girth. The plant biomass and dry weight were measured after 6 weeks of the experiment.

Data Analysis:

Data obtained was subjected to descriptive statistics and analysis of variance (ANOVA) using statistical package for social science (SPSS). Means were separated using Duncan multiple range test.

RESULTS

Three bacterial (*Pantoea eucarina*, *Bacillus wiadmannii* with percentage identity of 99.56 and 97.72%) and two fungal species of *Aspergillus flavus* with percentage identity of 99.64 and 99.82% respectively were identified by molecular methods Table 1 using gel electrophoresis Fig. 1. The isolates had the potential to solubilize Potassium and Phosphate and the Potassium and Phosphate solubilization index of the fungal isolates were presented in Fig 2. There was no significant difference in the results of potassium and phosphate solubilization index of the fungal isolates. The potassium solubilization index of fungal isolates F1, F2 and F3 were 0.94, 0.941 and 0.99, respectively while their phosphate solubilization index were 0.39, 0.44 and 0.27, respectively. Potassium and phosphate solubilization index of the bacterial isolates is presented in figure 3 with *Pantoea eucaine* having the highest potassium and phosphate solubilization index (1.024; 0.26) followed by *Bacillus wiadmannii* (0.81); 0.23 and least by *Aspergillus flavus* (0.55; 0.22) respectively.

The result of the phytohormone revealed that fungal isolates F1, F2 and F3 had 0.78, 1.43 and 1.03mg/l indole acetic acid (IAA) and 0.11, 0.17 and 0.17 mg/l gibberellic acid (GA)respectively and there was no significant difference between the two hormones produced by the isolates ($P \leq 0.05$) fig.3. Production of indole acetic acid and gibberellic acid by the bacterial Isolates followed similar trend. Indole acetic acid of the bacterial isolates (A1, A2 and A3) were 2.41, 2.54 and 2.59 mg/l while that of gibberellic acid were 1.09, 1.40 and 1.3 mg/l, respectively with no statistically significant difference ($P \leq 0.05$).

The effects of the isolates on the growth of maize after six weeks of planting showed that the plants applied with NPK had the highest increase in height, followed by the one inoculated with the mixed cultures of *P. eucarina* and *B. wiadmanni* and then the plant inoculated with *Pantoea eucarina*. There was no significant difference ($P \leq 0.05$) in the number of leaves produced by the tested plants (inoculated plants) and the control ones The highest plant biomass and plant dry weight were observed on the plant applied NPK with plant biomass and plant dry weight of 118.9 and 60.89g, respectively while the least was recorded on the control with plant biomass and plant dry weight of 35.32 and 15.27g, respectively.

Table 1: Molecular identity of the selected isolates

S/N	Sample ID	Blast Result	% Identity
1	Bacteria 1 (A1)	*Pantoea eucarina*	99.56%
2	Bacteria2 (A2)	*Bacillus wiadmannii*	97.72%
3	Fungi1 (F1)	*Aspergillus flavus*	99.64%
4	Fungi3 (F3)	*Aspergillus flavus*	99.82%

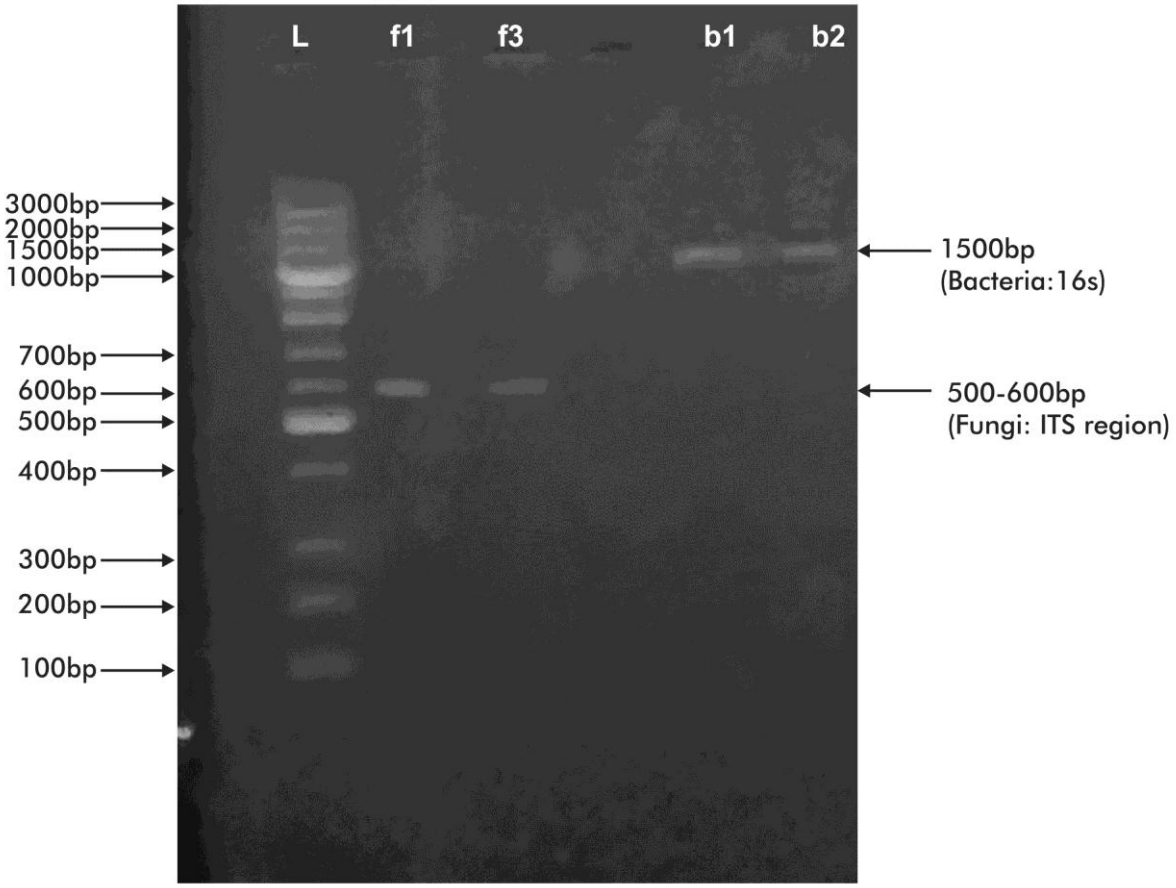

Fig 1 : Gel electrophoresis of 16sr RNA Amplicon

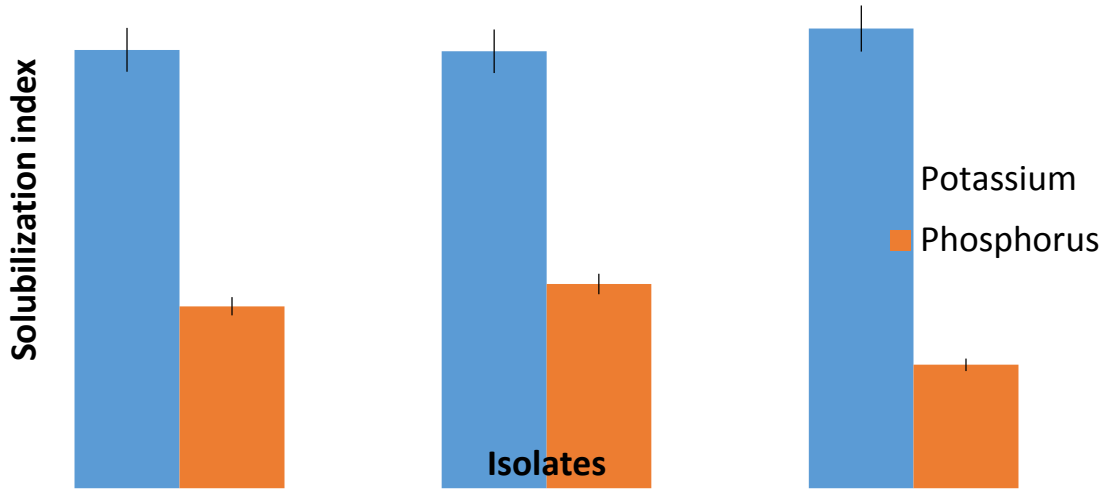

Fig 2: Potassium and Phosphate solubilization index by the fungal isolates

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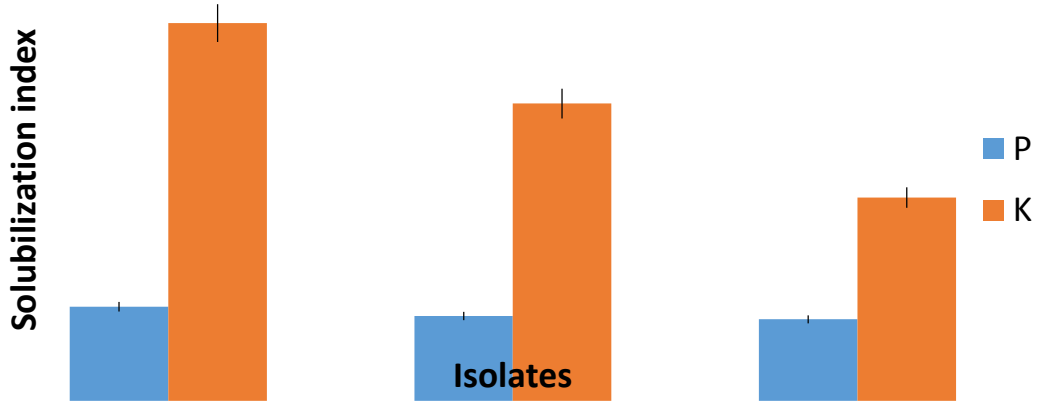

Fig 3: Potassium and Phosphate solubilization index of the bacterial isolates

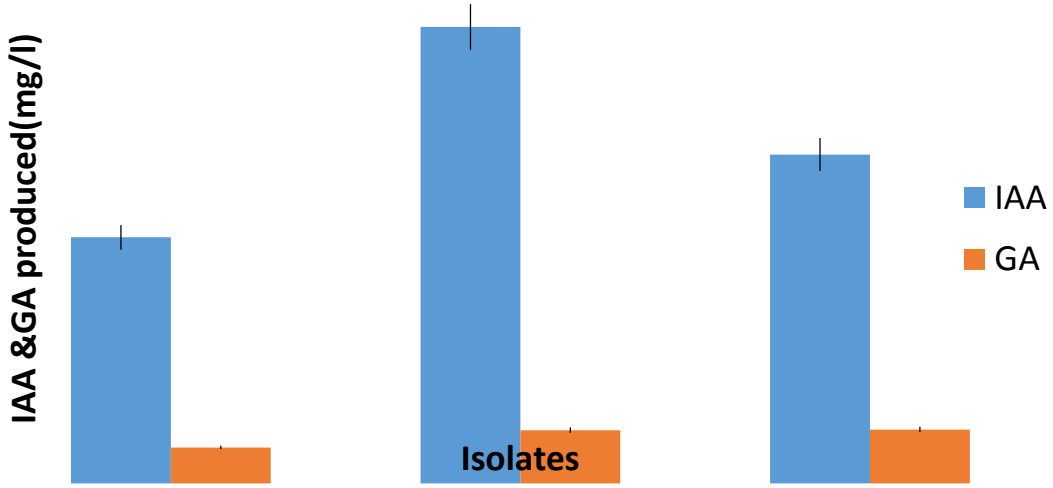

Fig 4: Indole acetic acid and gibberellic acid production by fungal isolates

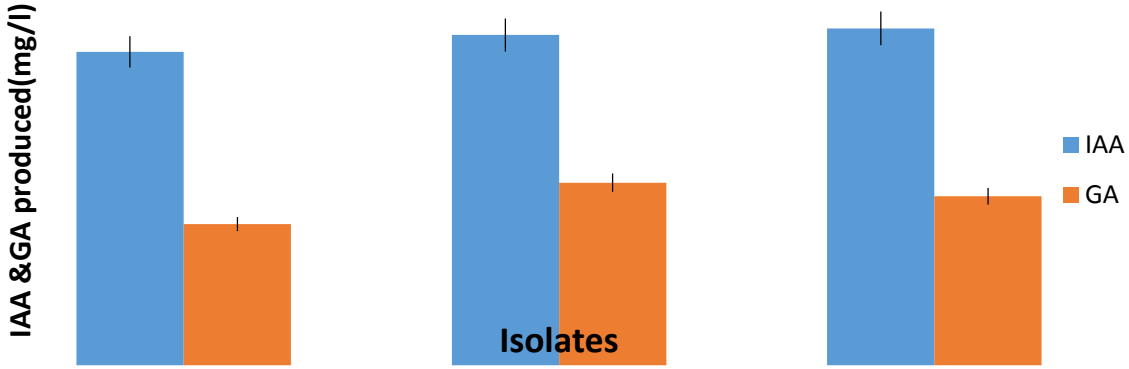

Fig 5: Indole acetic acid and gibberellic acid production by bacterial isolates

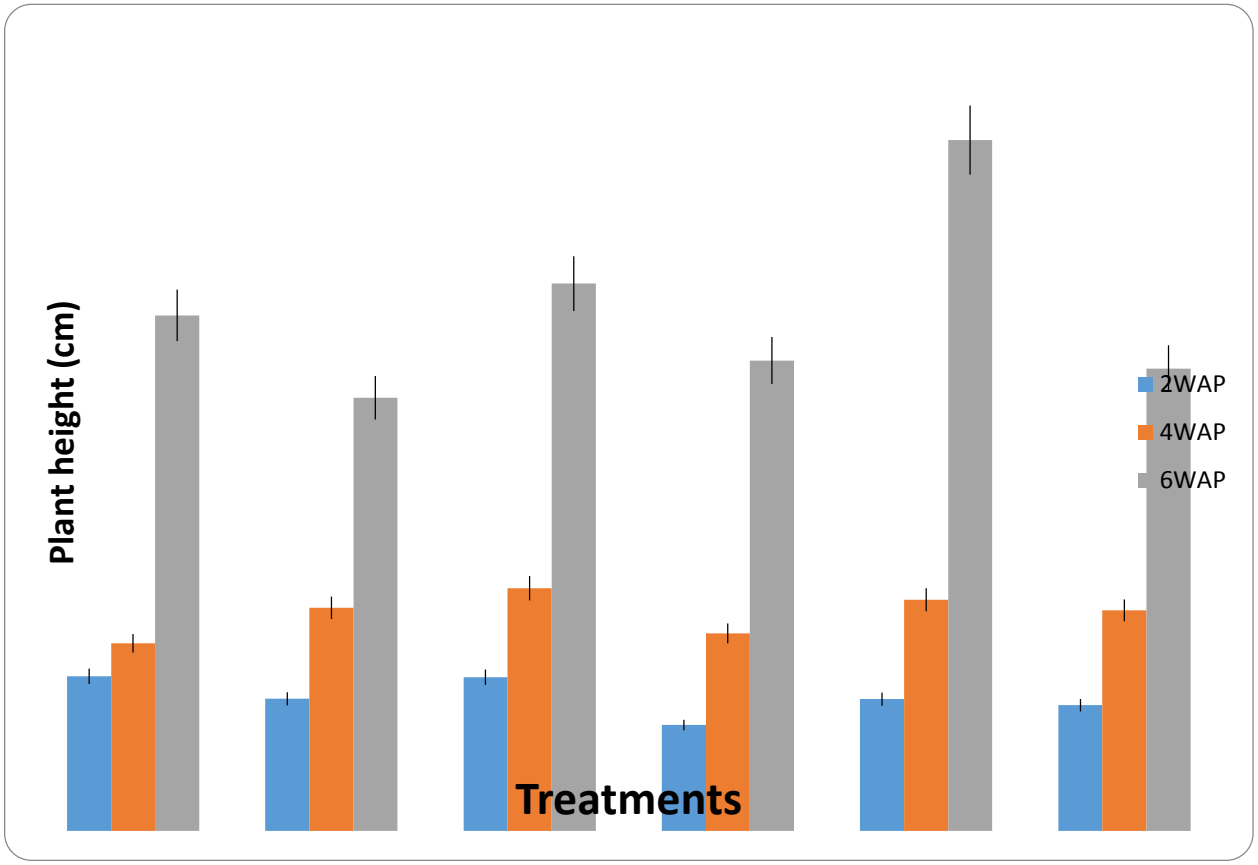

Fig 6: Effects of the isolates on plant height

Table 3: Effects of the isolates on the number of leaves

Isolates	Weeks after planting		
	2WAP	4WAP	6WAP
Pantoea eucaria	4.0±0.00a	4.3±0.03a	4.3±0.03a
Bacillus wiadmannii	4.0±0.00a	4.3±0.01a	4.6±0.03a
Pantoea eucaria	4.0±0.00a	4.7±0.03a	4.7±0.02a
+ Bacillus wiadmannii			
CONTROL	4.0±0.00a	4.0±0.00a	4.0±0.03a
NPK	4.0±0.00a	4.3±0.03a	5.0±0.00a
Fungi	4.00±0.00a	4.0±0.00a	4.0±0.00b

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DISCUSSION

Continuous and indiscriminate use of chemical fertilizers have led to loss of fertility and soil degradation with resultant low soil fertility and poor yield (Savci, 2012). The bacterial and fungal isolates used in this study showed significant phosphate and potassium solubilization index which is in agreement with the findings of Tan *et al.* (2014) who reported a significant solubilization of K and P by rhizobacteria. Akintokun *et al.* (2019) also reported a significant potassium and phosphate solubilization index by bacteria isolated from the rhizosphere of maize. The outcome of this study is also in line with the findings of Akintokun and Taiwo, (2016) who reported significant solubilization of phosphate and potassium by rhizobacteria isolated from the rhizosphere of tomatoes.

Quantification of P and K-Solubilized from insoluble source of phosphate and potassium by the isolates further revealed that the isolates can be of help in making phosphate and potassium available to plant through solubilization of insoluble form of phosphate and potassium in the soil. The solubilization could be attributed to the release of organic acid which solubilizes the insoluble phosphate (Panhwar *et al.,* 2012). The use of these isolates is necessary in order to minimize the excessive use of inorganic fertilizers in the soil as earlier stated by Sitepu *et al.* (2006). Tan *et al.* (2014) reported that the inorganic phosphate solubilisation was directly related to the production of organic acids and acid phosphatase by the microbes. This result agrees with the findings of Shekila *et al.* (2017) who reported a wide range of P solubilization by bacteria associated with medicinal plant. It was also consistent with the findings of Akintokun *et al.* (2019) who reported solubilization of insoluble organic and inorganic forms of phosphate and potassium by rhizobacteria isolated from the rhizosphere of maize.

Quantitative screening of the isolates showed the ability of the isolates to produce indole acetic acid and gibberelic acid. Production of IAA and GA is known to have a pronounced effect on plant growth and development. The results of our findings agreed with that of Parth and Bharatkumar, (2014) who reported the production of indole acetic acid by 80% of bacteria isolated from plant rhizosphere. Akintokun *et al.* (2019) also reported IAA and GA production by rhizobacteria isolates. The production of IAA and GA by the isolates is not unconnected to the root exudates which are natural sources of L-tryptophan which serves as precursor of IAA production. Production of IAA by the isolates is believed to be very crucial for the promotion of plant growth.

Tandya and Desai, (2014) reported GA production by bacterial isolates from the rice field. Umi *et al.* (2014) also revealed GA production by endohytic bacteria from rubber plants. The results of this work have shown that the native bacterial and fungal isolates could be useful for biofertilizer development. There is need for the farmers to use this native isolate as bio fertilizer so as to exploit the potential benefits associated with these isolates and as well reduce pollution due to over dependence on chemical fertilizers.

CONCLUSION

The isolates used in this study has proven to have a potential of been used in place of chemical fertilizer. This has been shown through the ability of the isolates to solubilize insoluble potassium and phosphorus source as well as produce phytohormones. The solubilization of insoluble potassium and phosphorus will help make them available to plant there by reducing deficiency of potassium and phosphorus in the soil. The phytohormones produced will also help to

enhance growth and development of plants thereby increasing crop yield.

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