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Assessment Of Phosphate Solubilization, Indole Acetic Acid and Ammonia Production By Bacteria And Fungi Isolated From The Rhizosphere Of Guinea Corn (*Sorghum bicolor*)

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Abstract

The rhizosphere is known to harbour a number of bacteria and fungi that can improve plant growth and productivity. This study investigated the abilities of bacteria and fungi isolated from the rhizosphere of guinea corn to solubilize phosphate, Idole Acetic Acid (IAA) and ammonia production. Purified isolates were confirmed through biochemical test and Gram staining. Phosphate solubilization was quantified using Pikovskaya's, medium on standardized isolates using standard curve. IAA, production was observed using spectrophotometer and ammonia production was confirmed using Mcfaland standard. Serratia marcescens, exhibited the highest phosphate solubilization activity (547.52 ug/ml), while Micrococcus luteus, exhibited the least (1.45 ug/ml). However, Escherichia coli, had the highest (1821.4 ug/ml), IAA, production and Serratia marcescens had the least (100.71ug/ml). Bacillus licheniformis, had the highest ammonia production (2452.65 ug/ml), while Lactobacillus bulgaricus, had the least (1495.77 ug/ml). Among the fungal isolates, Aspergillus niger had the highest phosphate solubilization effects (679.31ug/ml), while Mucor pusillus had the least ((1.82 ug/ml). Ammonia production was observed from all isolates screened. B. lichiniformis had the highest (2452.65ug/ml) and L. bulgaricus, had the least (1495.77ug/ml). The findings imply that rhizosphere soil of guinea corn harbours bacteria and fungi which can help to improve the availability of solubilized phosphate, production of IAA and ammonia.

Keywords: Rhizosphere, phosphate solubilization, IAA, Guinea corn

INTRODUCTION

Guinea corn also known as sorghum is a cereal grain that originated in Africa and it is now consumed throughout the world. It is an important food crop in Africa, Central America, and South Asia. It is the fifth most important cereal crop grown in the world (Abiala *et al.*, 2015). It is a nutrient-rich grain that is often ground into flour to make bread, porridge and pancakes and "Kunun drink" (Aliyu and Oyeyiola, 2011). Guinea corn contains about the same and sometimes more protein than many other grains. It is use for food, fodder, and the production of alcoholic beverages (Abiala *et al.*, 2015). However, guinea corn face serious threats of disease, partially due to the use of cultivars susceptible to diseases that are causing substantial yield losses (Ahmed *et al.*,2008). The excessive use of chemical pesticides has contaminated soils with adverse effect (Azizpour and Rouhrazi 2016). Accordingly, the use of biocontrol agents which are environmentally friendly are recommended for guinea corn disease control (Amusa and Odumbuka, 2009).

The root exudates in the rhizosphere provide a source of carbon and energy for microorganisms, increasing microbial biomass and their activity in the rhizosphere (Imam, 2008).

These include organic compounds called secondary metabolites produced by microorganisms during the alteration of primary metabolites (Kilic-Ekici and Yuen, 2003).

Bacteria that live in the rhizosphere that have a globally beneficial effect on plant growth are called plant growth-promoting rhizobacteria (PGPR).

The number of bacterial species identified as PGPR has recently increased as a result of numerous studies of a wider range of plant species, as well as advances in bacterial taxonomy and understanding of the various mechanisms of action of PGPR. Currently, PGPR includes representatives of a wide variety of bacterial taxa (Lucy *et al*., 2004). A variety of PGPR strains have been successfully used to inoculate crops. They are made up of members of the genus *Azospirillum* (Cassán and Garcia, 2008) Therefore, to prevent the use of synthetic pesticide and improve guinea corn plant growth, the use of this plant growth-promoting rhizobacteria (PGPR) offer the best alternative to colonize the roots and express plant growth promotion activities in the rhizosphere (Nwachoko and Alum, 2014).

MATERIALS AND METHODS

Study Area

The study was conducted in Niger State Polytechnic, Zungeru, located at Wushishi local government, Niger State. Niger State lies on longtitude 3.20° East and 11.30° North. Kaduna State and FCT, borders to the North-East and South-East of Niger State respectively; Zamfara State to the North, Kebbi State to West, Kogi State to the South and Kwara State to South West, while the republic of Benin along Agwara LGA borders her North West (Mohammed, 2002).

Collection of guinea corn seed samples

Red guinea corn variety was obtained from Institute of Agricultural Research Ahmadu Bello University, Zaria, Mokwa branch, Niger State, Nigeria. It was collected into clean polythene bag at ambient temperature of $25^0C \pm 3^0C$ before planting and transported to the microbiology laboratory of Federal University of Technology Minna, unopened.

Collection of rhizosphere soil samples

Rhizosphere soil samples were carefully collected by uprooting each plant using sterile trowel and the roots were shaken to obtain the soil adhering to the roots into clean polythene bags, labeled and transported from the field (farm) to the laboratory immediately for analysis.

Isolation of rhizosphere microorganisms

The rhizosphere soils were serially diluted to 10^{-3}, 10^{-4} and 10^{-5} in test tube containing 9ml of sterile distilled water each. Bacteria and fungi were isolated using nutrient agar and potato dextrose agar respectively. The inoculated plates were incubated accordingly. Subculturing of the isolate was done in order to obtain pure isolates (Chessbrough, 2012).

Isolation and enumeration of bacteria from the soil samples

Pour plate technique was used.to isolate bacteria from the unplanted soil during the period of cultivation of Guinea corn. One gramme of the soil sample was weighed and introduced into 9ml of sterile distilled water in test tube. The soil suspension was shakened and serially diluted to achieve dilutions. A 0.1ml aliquot was introduced from the different dilutions into separate plates. This was followed by the addition of sterile molten nutrient agar which was cooled to 43°C before pouring. The plate was swirled in other to homogenized the inoculum and the medium. The medium was then allowed to cool and incubated upside down at 37°C in an incubator for 24 to 48 hours. At the end of incubation period, the number of colonies on each plate was counted and expressed in cfu/g (Cowan and Steel's, 2001).

Purification and preservation of isolates

Representative colonies were selected on the nutrient agar plates and purified by subcultureing the dicrete colonies onto sterile nutrient agar. The plates were incubated at 37^0C for 24 hours (Fawole and Oso, 2001).

The non–filamentous fungi obtained were subcultured using inoculating loop to streak colonies on sterile. However, for the mycelial fungi, little portion of their growth was picked with the aid of sterile inoculating needle onto the surface of set plate of PDA and incubated at 25^0C for 72 hours. The process of subculturing was repeated until pure culture was obtained. The pure isolates obtained was transferred into sterile set plate of PDA and incubated at 25°C for 72 hours. The agar slants obtained were kept in the refrigerator at 4 – 8^0C until required for use (Dubey and Maheshwari, 2005).

Characterization of Bacterial Isolates

This was achieved by Gram staining and biochemical include; catalase, coagulase, oxidase, indole, utilization of citrate, fermentation of sugars, presence of spore, H_2S production of gas, oxidation fermentation test (OF), gelatin liquefaction, urease test, starch hydrolysis, triple sugar iron agar test, motility and methyl red-Voges Proskauer test (Peczar *et al*., 2005).

Spore staining of bacterial isolates

A smear of bacteria was made and heat fixed. The smear was flooded with 1% malachite green stain and heat with steam for five minutes. After five minutes it was drained, washed with distilled water and counter stain with safranine and stain for one minute. The stain was washed with distilled water and air dried. It was then viewed at x100 objective lens using binocular microscope. The endo spore appeared green and the vegetative cell appeared redish in colour (Chesbrough, 2012).

Biochemical tests of the isolates

Coagulase test

A drop of physiological saline was made on end of slide and colony of test organism was emulsify in each of the drops to make two thick suspensions. A drop of plasma was added to one of the suspensions and was mixed gradually. Appearance of coagulation (clumping) was observed which indicate positive (Cowan and Steel's, 2001).

Catalase test

The test bacteria colony was picked and emulsify with 3% hydrogen peroxide on a clean grease free glass slide A and B (B for control). The organism was only dropped in A. Bubble formation was observed which indicate positive. (Cowan and Steel's, 2001)..

Indole test

Bacterial isolates were inoculated with the pepton water and incubated at 37^0C for 48 hours. 0.5ml of Kovac's reagent was then added to the mixture in Mc Cantney bottle and gently shaked. Formation of a deep red ring on the top of the medium was observed (Fawole and Oso, 2001).

Urease test

Urea agar was prepared, inoculated with the test organisms and incubated at 37^0C for 24 hours. It was earlier observed to be red in colour and change of colour was observed to be pink which indicate positive. Negative result retained the initial orange colour of the medium (Cowan and Steel's, 2001).

Citrate utilization test

Simon citrate agar slant was prepared and test organisms were streak inoculated and incubated at 37^0C for 4 days. It was observed that the medium chanded from its original colour (green) to blue which means the reaction of the isolates was positive, while in a negative test, the media retained its initial green colour (Cowan and Steel's, 2001).

MR-VP (Methyl Red, Voges Proskauer)

Sterile glucose phosphate peptone water of 2ml was inoculated with the bacteria culture in in two seprate tube (A and B). Four (4) drops of methyl red reagent using pipette was added to tube A, mixed and immediately observed for colour production. Formation of pink colour between 2 – 5 minutes indicated a positive V P reaction (Cowan and Steel's, 2001).

Characterization of fungal isolates

The fungal isolates were characterized based on their macroscopic and microscopic characteristics. The macroscopic properties observed were size of colony, filamentousined surface colour of colony, nature of hyphae, types of asexual of spores either conidia contained in conidiaphore or spores contained in sporangiophore and colour of reverse side of culture plate. Likewise, appearance of septa in the hyphae, nature of vegetative hyphae such as presence of rhizoids their position and nature of spore whether rough or smooth (Fawole and Oso, 2001). In addition to the above, biochemical tests was performed to further assist in the identification of non-mycelial fungi. These include fermentation or utilization of 3% sugar such as galactose, sucrose, lactose, fructose, glucose, maltose and mannitol (Fawole and Oso, 2001).

Screening for Phosphate Solubilizing organisms

One gram (1g) of rhizosphere soil was suspended into 9 ml sterile distilled water in a tube and make serial dilutions up to 10^{-5}. Aliquot of 1ml suspension from 10^{-3} and 10^{-4} dilutions was added into agar plates containing Pikovskaya's agar medium which is supplemented with phosphate (calcium phosphate 15%). The plates were incubated at 25^oC for 4-5 days. Transparent zones of clearing around the colonies of microorganisms will indicate that phosphate has been solubilized or the presence of halo zone indicates phosphate solubilization (Imam, 2008).

Then, the culture was isolate, purify and identify it for quantitative determination of extent of solubilisation (Fawole and Oso, 2001).

Standardization of bacterial isolates

The bacterial isolates were standardized using 0.5Mcferland standard. This involved the preparation of 1% Barium chloride at 1% H_2SO_4. The 1% Bacl2 was prepared by adding 1.175g of $Bacl_2$ powder into 100ml of distilled water. One percent H_2SO_4 was prepared by adding 1ml of concentrated H_2SO_4 to 99ml of distilled water. Then, 0.5ml solution was removed from the 1% H_2SO_4 solution. Then 0.5ml of 1% $Bacl_2$ was added to the 99.54 of 1% H_2SO_4 remaining in the flask. The Turbidity was observed in the flask; this formed the 0.5Mcfarland solution (Cheessbrough, 2002).

Quantitative measurement of Phosphate Solubilization

Pikovskaya's liquid medium (100ml) was inoculated with 5ml of the standardized bacterial culture or 2 inoculum plugs from the advanced edge of 4 days old fungal culture. The broth culture was agitated at 120rmp for 15 days. Thereafter, the microbial culture was filtered through Whatman's filer No 42 and the filtrate centrifuged at 10,000 rpm. Barton's reagent was used for the phosphate quantification according to Dubey and Mashehwari (2005) (Cowan and Steel's, 2001). Optical density of the yellow-colored solution was measured using a spectrophotometer at 430nm wavelength. Amount of phosphate solubilized in culture medium was calculated by comparing using the standard curve.

Production of indole acetic acid (IAA)

The production of indoles (IAA) was tested according to the methods described by Aishiki *et al*. (2017). Microbial isolates were grown in their respective growth media, and was amended with 0.1% DL-tryptophan, and kept in a shaker incubator at 30°C and 180 rpm in the dark for 48 hours (bacteria) and 120 hours (Fungi). The liquid culture was then centrifuged at 8000 rpm for 10 minutes, and the supernatant was used to estimate indole production.

Two drops of orthophosphoric acid were added to 2 ml of supernatant and mix with 4 ml of Salkowski's reagent, which consists of 35% perchloric acid (50 ml) and 0.5 M $FeCl_3$ solution (1ml). The pink color formed in the reaction mixture indicated that the microbial isolate produced indole and absorbance was recorded at 530 nm wavelength using a UV-Vis spectrophotometer.

Quantification of ammonia production by the isolates

Twenty-four hours old culture of each bacterium was standardized using 0.5 McFarland. The standardized bacterial culture was inoculated (6%) into sterial peptone water (100ml) and placed in shaker orbital 120rpm at room temperature for 72hours. The culture was then centrifuged at 3000 rps for 30 minutes. One milliliter of the supernatant was added into 9ml of distilled water to obtain 10^{-1}. Furthermore, 1ml from 10^{-1} dilution was introduced into 50ml standard flask and 2ml of Nessler's reagent was added. The solution was allowed to stand for 30 minutes' absorbance read at 410 minutes. The amount of ammonia produced was obtained using ammonia standard curve (Aishiki *et al*. 2017).

Statistical Analysis

Statistical analysis included determination of the means, standard deviation, of mean, range etc. This was done using SPSS (version 21) Statistical package. Multiple Duncan range test was used to separate differences between the means 21).

RESULTS

Bacteria and fungi characterized and isolated from rhizosphere soil.

Bacteria isolated from rhizosphere soil of guinea corn include *Escherichia. Coli, Bacillus subtilis, Lactobacillus bulgaricus, Bacillus licheniformis, Micrococcus luteus, Pseudomonas aeruginosa, Pseudomonas fluorescens, Streptococcus faecalis, Serratia marcescens, Proteus vulgaris, Streptococcus pyogenes, Streptococcus pneumoniae, Bacillus megaterium and Klebsiella pneumoniae* (Table 1).

Fungi isolated from rhizosphere soil of guinea corn include: *Candida tropicalis, Fusarium oxysporum, Fusarium solani, Trichoderma person, Rhizopus stolonifera, Rhizopus oryzae Aspergillus niger, Mucor pusillus, Trichophyton quinckeneum, Trichophyton megininii, Aspergillus fumigatus, Aspergillus flavus, Verticillium tenerum and, Aspergillus terreus* (Table 2).

Table: 1 Characterization and identification of bacterial isolates

				Carbohydrate Utilization Test (Sugar Fermentation)													
G. Stai	Cat	Coug	SH	CIT	OXI	IND	MR	VP	H_2S	URE	HAE	L	S	G	M	Tentative Identity
- R	-	-	-	+	-	+	+	-	-	-	-	+	+	+	-	*Escherichia. coli*
+R	+	-	+	+	-	-	-	+	-	-	α	-	-	+	-	*Bacillus subtilis*
+R	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	*Lactobacillus bulgaricus*
+R	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-	*Bacillus licheniformis*
+C	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	*Micrococcus luteus*
-R	-	-	-	+	+	-	+	-	-	_	-	+	+	+	-	*Pseudomonas aeruginosa*
-R	-	-	-	+	+	-	+	-	-	-	-	+	+	+	-	*Pseudomonas fluorescens*
+C	-	-	-	-	-	-	-	-	-	-	α	-	+	+	-	*Streptococcus faecalis*
-R	+	-	-	+	+	-	+	+	-	+	-	+	+	+	-	*Serratia marcescens*
- R	-	-	+	+	-	-	+	-	+	+	-	+	+	+	-	*Proteus vulgaris*
+C	+	-	-	-	-	-	-	-	-	-	α	-	+	+	-	*Streptococcus pyogenes*
+	-	-	-	-	-	-	-	-	-	-	β	-	+	+	-	*Streptococcus pneumoniae*
+R	+	-	+	-	-	-	-	-	-	-	-	+	-	-		*Bacillus megaterium*
- R	+		-	+	-	-	-	+	+	+	-	+	+	-		*Klebsiella pneumoniae*

KEYS:

+R = Gram positive rods -R = Gram negative rods +C = Gram positive cocci
+ = Positive "has reaction" - = Negative "no reaction"
L = Lactose Sugar S = Sucrose sugar G = Glucose
M = Mannitol Sugar H_2S = Hydrogen Sulphide production Test URE = Urea Libration Test
Hae = Blood haemolysis Test α = Alpha haemolysis β = Beta haemolysis
γ = Gama G. Stain = Gram Stain Reaction Cat. = Catalase
Coug. = Coagulase CIT = Citrate Utilization Test OXI. = Oxidase
IND = Indole MR = Methyl Red V.P = Voges prauskar Test
SH = Starch hydrolysis

Table 2: Characterization and identification of the fungal isolates

S/NO	Fungal Isolates	Macroscopic characteristics	Microscopic characteristics	Tentative Identity
1	*F1*	Initially grey, some maintain the grey, some changes to yellow mat-like in shape	Single cell oval in shape, Blastospores, presence of Pseudomycelia	*Candida tropicalis*
2	*F2*	Initially white wooly; as it age it changes to yellow	Microconidia, Spindle shaped conidiophore	*Fusarium oxysporum*
3	*F3*	Initially white as it ages it changes to salmon white	Microconidia, Spindle shaped conidiophore	*Fusarium solani*
4	*F4*	Initially white as it age, it changes to light and bluish green wrinkled in shape	Conidia	*Trichoderma persoon*
5	*F5*	The colonies are yellow brown and thick wooly in nature	Sporangiospores	*Rhizopus stolonifer*
6	*F6*	They patchily light, black in colour thick wooly	Sporangiospores	*Rhizopus oryzae*
7	*F7*	Initially white as it age it changes to black	Conidia radiating from sterigma, Multinucleated conidia is contained in conidophore	*Aspergillus niger*
8	*F8*	Growth in less 24hrs and filled plate with wooly grow like cotton wool	Sporangium contain numerous spores. Has sporangiophore developed from mycelium	*Mucor pusillus*
9	F9	Initially show white later changes to brownish	Pear shaped microconidia. Long septate contain branded multinucleated microconidia	*Trichophyton quinckeneum*
10	*F10*	Whitish colony, spiral in shape	Oval or pear shaped conidia. Long, thin, smooth wall, multinucleated macroconidia with blunted end	*Trichophyton megininii*
11	*F11*	Colony initially white and change to green as it age	Conidia radiating from sterigma	*Aspergillus fumigatus*
12	*F12*	Colony initially white; and changes yellow	Conidia radiating from sterigma	*Aspergillus flavus*
13	*F13*	Colony initially white; and changes to red	2 – 6 unicellular elliptical conidia	*Verticillium tenerum*
14	*F14*	colony initially white; and changes to brown as it ages	Conidia radiating from sterigma	*Aspergillus terreus*

Phosphate solubilization effect of the fungal isolates

Among the fungal isolates from the guinea corn rhizosphere soil *Aspergillus niger,* had the highest phosphate solubilization effects (679.31ug/ml), while *Mucor pusillus* had the least ((1.82 ug/ml) as shown in table 3.

Table 3: Phosphate solubilization by the fungal isolates

Fungal isolates	Phosphate solubilized (µg/ml)
Trycophyton	3.63
Mucor pucillus	1.82
T. quickenum	6.16
A. fumigatus	13.08
Trichoderma persoon	252.71
Aspergilus flavus	125.17
Verticillium tenerium	154.36
Aspergilus niger	679.31
T. verrucosum	0.00

Phosphate solubilization effect of the bacterial isolates

Phosphate solubilization effects of bacterial isolates from the guinea corn rhizosphere soil are shown in Table 4. *Serratia marcescens,* exhibited the highest phosphate solubilization activity (547.52 ug/ml), while *Micrococcus luteus,* exhibited the least (1.45 ug/ml) phosphate solubilization effect.

However, for indole acetic acid production (IAA) (Table 4), *Escherichia coli,* had the highest (1821.4 ug/ml), production of IAA, where by *Serratia marcescens, had the least* (100.71ug/ml). Also, in ammonia production by the bacterial isolates, *Bacillus licheniformis,* had the highest production (2452.65 ug/ml), while *Lactobacillus bulgaricus,* had the least (1495.77 ug/ml)

Table 4: Phosphate solubilization, indole acetic acid and ammonia production by the Bacterial isolates.

	µg/ml		
Bacteria isolates	I.A. A	Phosphate solubilization	Ammonium producers
Bacillus subtilis	118.29	2.82	2108.79
Micrococcus luteus	100.86	1.45	2101.75
Serratia marcescens	100.71	547.52	1857.75
Escherichia coli	1821.4	455.57	1838.22
Lactobacillus bulgaricus	418.29	22.17	1495.77
Bacillus licheniformis	362.57	121.30	2452.65

DISCUSSION

Phosphorus is commonly present in forms that are not immediately available to plants. Due to the slow diffusion rate in the soil which often results in a zone of P-depletion around plant roots as P is absorbed faster than it can be made available to replenish the soil around the root (Bagayoko *et al.*, 2000).

Soil fungi and bacteria inhabiting around or on the root surface of plants directly involved in plant growth improvement and protects plants from disease and abiotic stresses conditions through the production of various regulatory chemicals in the vicinity of rhizosphere (Barea *et al.*, 2005). The use of phosphate solubilizing microorganisms as inoculants is one of the alternative biotechnological solutions in sustainable agriculture to meet the phosphate demands of plants (Liu *et al.*, 2014).

In the present study, *Escherichia coli, Bacillus licheniformis* and *Lactobacillus bulgaricus* isolated from rhizospheric soil exhibited phosphate solubilization effect (Table 2). The results indicated that *Serratia marcescens* are better phosphate solubilizer (547.52ug/mL) while *Micrococcus luteus* (1.45ug/ml) exhibited the least phosphate solubilizing effect (Table 2).

These results indicated that rhizospheric bacteria have the ability to solubilize precipitated phosphates as reported by (Solaimam and Anawar, 2015). According to Aishiki *et al.*, (2017). in addition to rhizobacteria, several fungi such as species of *Aspergillus* can efficiently solubilize P. This is in consistent with the results obtained in this study as *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Mucor pusillus, Trichophyton verrucosum, Trychophyton megininii* and *Trichophyton quinckeneum* were able to solubilize phosphate (Table 1). However, results obtained in this study indicated that fungal isolates were able to solubilize phosphates better than the bacterial isolates (Table 1). This finding disagrees with the study of (Raaijmakers *et al.*, 2009) which reported that even though the fungal isolates were able to solubilize phosphates to some extent; their ability was not comparable to that of the bacterial isolates. All of the above-identified fungi and bacterial species found to be efficient phosphate solubilizes which have a great role in increasing crops productivity and production without contaminating the environment and affecting human health (Verma *et al.*, 2001).

Production of indole acetic acid (IAA) was observed from six bacteria isolates, which include: *B. subtilis, M. luteus, S. marcescens, E. coli, L. bulgaricus and B. licheniformis*. Four of these isolates were gram positive while two are gram negative. This is inconformity with findings of (Mwajita *et al.*, 2013), which says IAA producing organism are mostly gram positive and few gram negative among them belong to class of Bacillus strain.

Lactobacillus bulgaricus had the highest IAA, production and *Serratia marcescens,* had the least production. Production capability of the isolates can be influenced by cultural condition, stage of growth and availability of substrate (Wahyudi *et al.*, 2011) made similar observations.

CONCLUSION

It is concluded that the rhizosphere soil of guinea corn harbours bacteria and fungi which can help to improve the availability of solubilized phosphate, production of IAA and ammonia. By indication, the rhizospheric *Seratia marcescens* and *Aspergillus niger* isolated in this study would enhance the growth of *Sorghum bicolor* plant and this would lower the use of chemical fertilizer and have greater potential application in the field.

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