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MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF ENDOPHYTIC BACTERIA FROM ROOT OF *Chamaecrista rotundifolia* GROWING ON THE NIGERIAN AIR FORCE SHOOTING RANGE, KADUNA.

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ABSTRACT

Chamaecrista rotundifolia is one of the few plant species growing on heavy metal-contaminated Nigerian Air Force shooting range, Kaduna. This microbially unexplored plant species can have microbial association with endophytes. This study was aimed at isolating, identifying and characterizing endophytic bacteria associated with the roots of *Chamaecrista rotundifolia* growing on the Nigerian Air Force shooting range, Kaduna. The identification of the endophytic bacteria was done using standard biochemical methods and 16S rRNA sequencing analysis. The isolates were then screened for antibiotic resistance, plant growth promoting properties and heavy metal tolerance using standard techniques. Varied concentrations of heavy metal salts (Pb^{2+}, Cr^{3+}, Ni^{2+} and Mn^{2+}) were used to test the isolates for their heavy metal tolerance. Four endophytic bacteria isolated from the roots of *C. rotundifolia* were *Aerococcus viridans, Peribacillus simplex, Staphylococcus simplex and Pseudomonas aeruginosa.* Results on antibiotic sensitivity test revealed that the four isolates were resistant to most of the antibiotics. The isolates possessed plant growth promoting properties by showing positive results for Indole-3-acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylic (ACC) deaminase activity and phosphate solubilizing. Results also revealed that the isolates showed some degree of tolerance to the heavy metals. The tolerance of both *A. viridans* and *P. simplex* to chromium and manganese showed significant difference ($p<0.05$) across all the concentration levels of the metal salts. It is therefore concluded that *Chamaecrista rotundifolia* growing on Nigerian Air Force shooting range, Kaduna, is associated with endophytic bacteria which could be the reason the plant species thrive in heavy metal-contaminated site.

KEYWORDS: *Chamaecrista rotundifolia,* Endophytic bacteria, Heavy metals, Tolerance.

INTRODUCTION

Heavy metal contamination from explosives during military training activities, is one of the major issues of global concern due to its deleterious effects on animals, plants and human health (Onyacha *et al*., 2008; Fan *et al*., 2016). Military shooting range harbors heavy metal contamination due to excessive shooting exercises involving the use of real cartridges, ammunitions and explosive chemicals. Saleh *et al*. (2004) and Mench (2009) reported that explosives contain some heavy metals such as Pb, Cr, Ni, As, and Mn that are recalcitrant, toxic and persistent in the environment for a long time and which in turn, affect plant distribution and diversity. During range operation and maintenance, this heavy metal contamination has been reported to cause serious environmental and occupational health hazards (Nwaedozie *et al*., 2015);

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and they are extremely toxic leading to mutagenic, carcinogenic, neurologic and teratogenic effects on biological systems (WHO, 2019). In recent years, phytoremediation assisted by plant growth promoting bacterial endophytes for cleaning up of heavy metal polluted soils, has been recommended (Das *et al*., 2014; Hao *et al*., 2015; Chen *et al*., 2016).

Endophytes are group of microorganisms (bacteria, fungi or actinomycetes) that dwell within robust plant tissues. They have the ability to enter inside the plant host and colonize plant tissues such as plant roots, leaves and stems or may form specific structures such as root nodules which depends upon the specific bacterium and the plant (Ali *et al*., 2014). Plant Growth Promoting Endophytes (PGPE) once established inside the plant tissue are no longer subject to the vagaries of soil condition, but depend on plant conditions such as plant health and nutritional state as well as the plant tissue analyzed (Ma *et al*., 2016). Nwaedozie *et al.* (2015) reported that Nigerian Air Force shooting range is contaminated with heavy metals such as lead (Pb), Chromium (Cr), Nickel (Ni) and Manganese (Mn) and it is characterized by acidic soils with the presence of four plant species: *Chamaecrista rotundifolia, Sida veronicifolia, Chrysanthemum americanum and Borreria filifolia*. The prevalence of these plant species in the heavy metal-contaminated shooting range shows that the plant species may be harboring some plant growth promoting endophytes (PGPE) which enable them to survive in the environment.

Chamaecrista rotundifolia was selected for this study because of its unique characteristics and efficiency compared to the three other plant species; these characteristics are growth habit (the plants continue to grow after flowering) and excellent seedling survival under harsh conditions (Sanderson *et al.,* 2010). *C. rotundifolia* growing at Nigerian Air force shooting range has been screened by Nwaedozie *et al.* (2015) and found to possess heavy metals in its roots but endophytic bacteria from the plant has not yet been studied extensively, hence the need for this study. Therefore, the main objective of this study is to molecularly identify and characterize endophytic bacteria from the root of *Chamaecrista rotundifolia* growing on the Nigerian Air Force shooting range, Kaduna.

MATERIALS AND METHODS

Study Area

The study was carried out at the Air Force shooting range located within the Nigerian Air Force Base, Kaduna. It lies between latitude 10º 36' 33.5484''N and longitude 7º 25' 46.2144''E. **Sample Collection**

Sterile technique was employed to collect root samples of *Chamaecrista rotundifolia* plants from the Nigerian Air Force shooting range. Three intact root system of the plant was dug out at different points in a given section and labelled properly according to the collection point and was placed on a dry cool place to avoid moisture accumulation or excessive drying.

The samples were carefully transported immediately to the microbiology laboratory of Nigerian Defence Academy, Kaduna, in sterile polyethylene bags and stored at 4°C for identification, biological assays and isolation of endophytic bacteria.

Isolation of Endophytic Bacteria from Root Samples

The rhizome pieces of the native plants were washed with tap water to remove soil and cut 1-2 cm long pieces. The root samples were dipped in 70 % ethanol for 1 min to sterilize the root samples, washed several times with distilled water and the final wash was spread plated onto nutrient agar plate and used as control. For the isolation of endophytic bacteria, the outer surface of the sterilized plant material was trimmed, the pieces further macerated in Phosphate buffer saline (PBS) and serially diluted up to 10^{-5} (Glick, 2015). Nutrient agar plates were prepared and 100µg/ml of Nystatin was added to the media to prevent the growth of fungi. Then, 1ml from 10^{-5} dilution was plated onto center surface of sterilized nutrient agar plates in triplicates. All plates including the control were incubated at room temperature for 48 hours and observed periodically for bacteria growth. Morphologically distinct colonies as identified by colony characters were selected, purified and used for further studies (Glick, 2015).

Bacterial Enumeration

The Colony Forming Unit (CFU) was calculated by counting the viable cells on each plate after 24 hours of incubation. The average colony count of the plates was calculated and the Colony Forming Unit (CFU) was then calculated using the formula:

$$\text{CFU/mL} = \frac{No\ of\ Colonies \times Dilution\ factor}{Volume\ of\ culture\ plate}$$

Single colonies were picked using a loop and purified by streaking repeatedly on MacConkey agar (a selective and differential media used for the isolation of gram negative, enteric bacilli and lactose fermenting organisms), chocolate agar (differential medium for gram positive cocci and a non-selective medium that supports the growth of both fastidious and non-fastidious organisms) and blood agar (selective for fastidious gram negative organisms such as *Pseudomonas*) at room temperature for 3 days in order to obtain pure bacterial isolates. Pure isolates were stored on nutrient agar slant in the refrigerator at 4ºC (Chen *et al*., 2016).

Morphological and Biochemical Characterization of the Isolates

The isolates were identified morphologically for their colony appearance, colour and shape. Standard methods were used to carryout biochemical tests to characterize the isolates.

Gram Staining

A thin smear of each of the pure 48-hour old pure culture were prepared on clean grease-free slides, fixed by passing over gentle flame, stained by

addition of 2 drops of crystal violet solution for 60 seconds and then, rinsed with water. The smear was again flooded with Lugol's iodine for 30sec and rinsed with water, decolorized with 70% alcohol for 15sec and rinsed with distilled water. It was counter-stained with 2 drops of safranin for 60sec and finally rinsed with water. The smears were mounted on a microscope and observed under oil immersion objective lens. Gram negative cells appeared pinkish-red while gram positive organisms appeared purple (Cheesbrough, 2006).

Catalase Test

Small quantity of each pure isolate culture was transferred into a drop of 3% Hydrogen peroxide solution on a clean slide with the aid of sterile inoculating loop. Gas seen as white froth indicated the presence of catalase enzyme (Cheesbrough, 2006).

Nitrate Reduction

Some quantity of each pure isolate was inoculated into nitrate broth using aseptic technique. Then, one dropperful of sulfanilic acid and one dropperful of α-naphylamine were added to the broth. A colour change to red indicated a positive nitrate reduction test (Cheesbrough, 2006).

Citrate Utilization

Each isolate from nutrient agar was picked up with a straight wire, then inoculated in simmon´s citrate agar and incubated at 37ºC and examined daily. A positive test was indicated by change of colour from green to blue (Cheesbrough, 2006).

Motility Test

Sterile needle was used to pick a loop of pure isolated culture from each plate and stabbed onto nutrient agar in glass vials. The vials were incubated at 37ºC for 48hr. Non-motile bacteria had growth confined to the stab line with definite margins without spreading to the surrounding areas while motile bacteria gave a diffused growth extending from the surface (Olutiola *et al.,* 2000).

Indole Test

The test cultures were inoculated into peptone water medium and incubated at 37ºC for 48 hours. Then, 1ml of Kovacs´s reagent was run down to the side of the tube. A pink ring which appeared on the surface within 1 minute indicated positive reaction (Cheesbrough, 2006).

Methyl Red Test

The test organisms were inoculated in glucose phosphate peptone water, incubated at 37ºC for 2 days. Five drops of methyl red reagent were added. A positive reaction was indicated by appearance of a red colour (Cheesbrough, 2006).

Spore Staining Technique

Smears of the isolates were prepared on separate slides and flooded with 5% malachite green solution and steamed for a minute. The stain was washed off with water and counter-stained with 2 drops of safranin solutions for 20sec. The slide was allowed to air dry and examined under oil immersion objective (×100) lens. Endospores stained green while vegetative cells stained pink (Cheesbrough, 2006).

Oxidase Test

A piece of filter paper was soaked with few drops of oxidase reagent. Sterile inoculating loop was used to pick each colony of the test isolates and smeared on the filter paper. A deep purple colouration indicated positive test. (Cheesbrough, 2006).

Molecular Characterization of the Isolates using 16S rRNA

DNA of each isolate was extracted using phenol chloroform standard method as described by Kochl *et al*. (2005) in which 200µl was added in a 1.5ml microcentrifuge tube; and then, 400 µl of lysis buffer, 10µl of proteinase k, 400µl of phenol, 400µl of Chloroform were added into the tube and vortexed. The extracted DNA from bacteria was quantified using Nano-drop spectrophotometer. The 16S Primer Forward 5'-GGACTACAGGGTATCTAAT-3' and 16S Primer reverse 3'-AGAGTTTGATCCTGG-5' was used for the amplification in a programmable thermocycler whose operation conditions are: pre-denaturation at 90°C for 5min, 25 cycles of denaturing at 94°C for 1 min, annealing at 52°C for 1 min extension at 72°C for 1 and final extension at 72°C for 7 min (Amin *et al*., 2012). The amplified PCR products were run on 1.5% agarose gel electrophoresis stained with ethidium bromide and viewed under ultra violet (UV) light (Black and Foarde, 2007). The amplified products were sequenced using DNA sequencing machine (ABI PRTSM™ 310 Genetic Analyzer) All the sequences were matched against nucleotide sequences present in Gen Bank using the Basic Local Alignment Search Tool (BLAST) NCBI (National Center for Biotechnology Information) program to identify the organism based on the most similar 16SrRNAgene.

Antibiotic Resistance Test

Disc diffusion method was used to examine the bacteria susceptibility to antimicrobial agents. The antibiotic sensitivity discs utilized were: perfloxacin (5µg), Septrin (30µg), Nalidixic acid (10µg), Amoxacillin (30µg), Gentamycin (10µg), Ampiclox (30 µg), (30µg) Streptomycin (25 µg), Erythromycin (15µg) and ciprofloxacin (10 µg) with isolate incubated at. After incubation at 37ºC for 24hr, a clear zone of inhibition around an antimicrobial disc reflects the degree of susceptibility of the organism to the drug (Mahon *et al*., 2007). The growth of the bacterial colonies was observed and zone of inhibition measured. The diameter of the inhibitory zones exhibited by the bacterial isolates against the respective antibiotics were then translated into resistance and susceptibility categories according to the National Committee for Clinical Laboratory Standards (NCCLS, 2004).

Screening of the Isolates for Plant Growth Promoting Properties

Indole-3-Acetic Acid (IAA) Production

The bacteria isolates were inoculated into 20ml of nutrient broth supplemented with 0.2 % (v/v) of L-

tryptophan and incubated for 7 days at 28ºC. After incubation, the cultures were centrifuged at 3000 rpm for 20 mins and the supernatants were used for analyzing indole-3-acetic acid production. One ml of the supernatant was added to 2ml of Salkowski reagent in a tube and incubated in the dark for 30 min at 25ºC. The development of pinkish or red color indicated positive result. The absorbance of the final solutions was measured at 530nm and the concentration of IAA in the culture medium was determined using the standard curve of pure IAA (Zhang *et al.,* 2011).

Aminocyclopropane-1-Carboxylic Acid (ACC) Deaminase Production

The ACC deaminase production of the isolates was screened using the methods described by Jasim *et al.* (2013). The isolates were inoculated on to differential salts minimal medium amended with 0.2 % ammonium sulphate (w/v). The bacterial growth in this media after 2 days of incubation was considered as positive result.

Phosphate Solubilization

The isolates were screened for phosphate solubilization using the procedure described by Jasim *et al.* (2013). Pikovskaya medium containing 2.4 mg/mL bromophenol blue was used. The media inoculated with the isolates were incubated for 48 hrs and observed for the formation of yellow zone around the colony due to the utilization of tricalcium phosphate present in the medium.

Determination of Heavy Metal Resistance of the Isolates

Minimum inhibitory concentrations (MIC) of the metals were determined by the Agar diffusion method as described by Velusamy *et al*. (2011).

Minimum Inhibiting Concentration (MIC) is defined as the lowest concentration of the metal inhibiting the visible growth of the bacteria. The isolates were tested for their resistance to heavy metals such as Pb^{2+}, Cr^{3+}, Ni^{2+} and Mn^{2+} [$Pb(NO_3)_2$, $CrCl_3.6H_2O$, $NiCl_2$ and $MnCl_2$ salts respectively]. Nutrient agar medium was prepared, autoclaved, and allowed to cool to 50ºC. The pH of the medium was adjusted to 7.0. Stock solutions of each heavy metal salts were prepared in distilled water in the concentrations of 0,05 mg/L, 0.15 mg/L, 0.25 mg/L and 1mg/L, and then added on the nutrient agar plates. A positive control consisted of a metal deficient medium inoculated with the isolate. A negative control consisted of a metal-supplemented medium without the isolate. The drop-inoculated plates were then incubated at room temperature (37˚C) for 24-48 hrs. Zones of inhibitions (mm) were measured after 24 hrs to the 7th day of incubation using a meter rule.

Statistical Analysis

Data obtained from the study was subjected to statistical analysis using Software Statistical Package for Social Scientist (SPSS) software version 22.0. One-way Analysis of variance (ANOVA) was used to compare the mean of the heavy metal tolerance of bacteria isolates at 0.05 significant level ($P<0.05$).

RESULTS

Morphological Features of the Endophytic Bacterial Isolates of *C. rotundifolia*

A total of four endophytic bacteria were isolated from the root of *C. rotundifolia;* and were coded as: CR1 for *Aerococcus viridans,* CR2 for *Peribacillus simplex,* CR3 for *Staphylococcus epidermis,* CR4 as *Pseudomonas aeruginosa*. (Table 1). CR1 was characterized by small circular colonies with yellowish white colour. CR2 and CR4 were by rod shaped and had foul smell, while CR3 is characterized by spherical clustered shape with yellowish golden colour and foul smell.

Table 1: Morphological Characterization of Endophytic Bacterial isolates of *C. rotundifolia*

Morphological Features	CR1 (*Aerococcus viridans*)	CR2 (*Peribacillus simplex*)	CR3 (*Staphylococcus epidermis*)	CR4 (*Pseudomonas aeruginosa*)
Colony shape	Small round clusters	Rod	Grape-like spherical clusters	Rod
Colour	Yellowish white	Off white	Yellowish golden	Yellowish white
Edges	Clustered	Jagged	Comets	Rough
Smell	Foul-smell	Foul smell	Foul smell	Grape-like

Biochemical Characterization of the Endophytic Bacteria Isolates of *C. rotundifolia*

The result of biochemical tests as presented in Table 2, revealed that isolates CR1, CR2 and CR3 were gram positive while CR4 was gram negative. Only CR2 and CR4 were positive to motility test. All the isolates were positive for citrate utilization and negative for indole test. CR1 and CR3 were positive to methyl red test and CR4 and CR5 were positive for oxidase test.

Table 2: Biochemical Characterization of the Endophytic Bacterial Isolates of *C. rotundifolia*

Biochemical Test	CR1 (*Aerococcus viridans*)	CR2 (*Peribacillus simplex*)	CR3 (*Staphylococcus epidermis*)	CR4 (*Pseudomonas aeruginosa*)
Gram stain	+	+	+	-
Motility Test	-	+	-	+
Indole Test	-	-	-	-
Citrate Utilization	+	+	+	+
Methyl red	+	-	+	-
Spore forming	-	+	-	-
Oxidase	-	-	-	+

- = Negative; + = Positive

Molecular Characterization of the Endophytic Bacteria Isolates of *C. rotundifolia*

Agarose gel electrophoresis pattern of the bacterial isolates showing PCR amplified products of 16S rRNA gene with band size 800bp is shown in Fig. 1. The sequence obtained for the gene of each isolate was compared against the non-redundant nucleotide sequence collected at NCBI Genbank using the web interface of NCBI-BLAST. BLASTn analysis of the PCR product indicated that the organisms were identified as CR1 -*Aerococcus viridans* CCUG4311, CR2 - *Peribacillus simplex* NBRC15720, CR3 - *Staphylococcus epidermis* ATCC:14990 and CR4 - *Pseudomonas aeruginosa* PA01 (Table 3). Phylogenetic tree demonstrates that CR2, CR3, and CR4 are all descended from the same ancestral lineage; with CR3 and CR4 being more closely related than CR2, which evolved after speciation through divergence. This demonstrates that the CR2 sample is more closely linked to the other organism since they share a most recent common ancestor (Fig. 2).

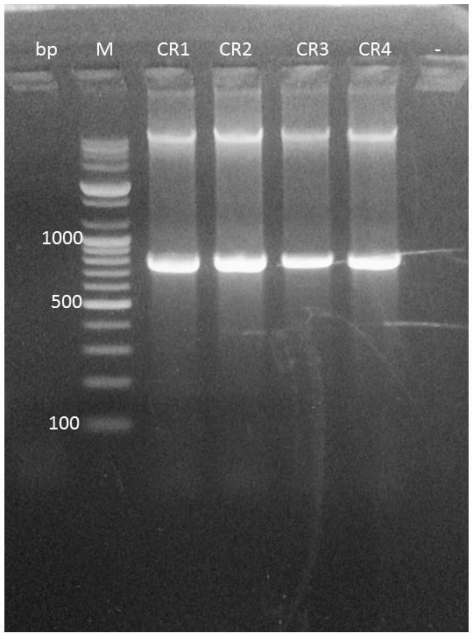

Fig. 1: Agarose gel electrophoresis pattern showing PCR amplified products of 16S rRNA

Table 3: Sequencing and BLAST Analysis of the Isolates

Isolates	Accession Number	Length	Identity percentage	Identified Organism	Bacterial Strain
CR1	NZ_CP014164.1	2199877	98.08%	*Aerococcus viridans*	CCUG4311
CR2	NZ_CP017704.1	5645783	97%	*Peribacillus simplex*	NBRC15720
CR3	NZ_CPO35288.1	2466502	100%	*Staphylococcus epidermis*	ATCC:14990
CR4	NC_002516.2	6264404	82%	*Pseudomonas aeruginosa*	PA01

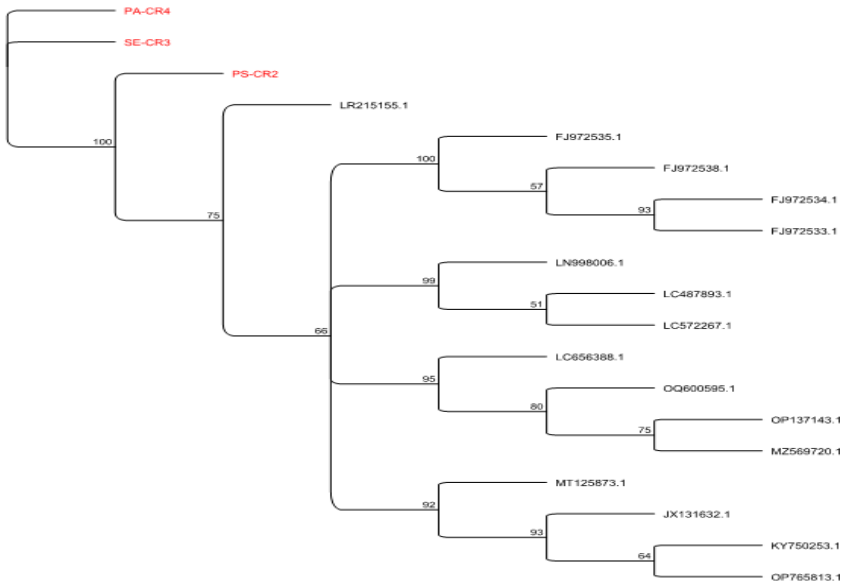

Fig. 2: Phylogenetic tree of the bacterial isolates.

Antibiotic Resistance

The result of antibiotic resistance test as shown in Table 4 revealed that CR1 is resistant to other antibiotics except ciprofloxacin and streptomycin. CR2 is sensitive to only gentamycin and erythromycin while CR3 is sensitive to ciprofloxacin, ampiclox, gentamycin, perfloxacin and streptomycin. CR4 is sensitive to only ciprofloxacin.

Table 4: Antibiotic Resistance of the Endophytic Bacterial Isolates

Isolate	RD (10g)	CPX (10g)	AM (5g)	APX (30g)	SXT (10g)	CN (10g)	E (15g)	PEF (5g)	ST (25g)
CR1	R	S	R	S	R	R	R	R	S
CR2	R	R	R	R	R	S	S	R	R
CR3	R	S	R	S	R	S	R	S	S
CR4	R	S	R	R	R	R	R	R	R

R = Resistance, S = Sensitive, RD = Nalidixic acid, CPX = Ciprofloxacin, AM = Amoxacillin, APX = Ampiclox, SXT = Septrin, CN = Gentamycin, E = Erythromycin, PEF = Perfloxacin, ST = Streptomycin.

Plant Growth Promoting Properties of the Isolates

The isolates possessed plant growth promoting properties, showing positive results for Indole-3-acetic acid (IAA) production, 1-aminocyclopropane-1-carboxylic (ACC) deaminase activity and phosphate solubilizing. CR3 (*Staphylococcus epidermis*) showed a negative result for ACC deaminase test (Table 5).

Table 5: Plant Growth Promoting Properties of the Isolates

Bacterial Isolate	IAA Production	Phosphate solubilization	ACC Deaminase
CR1	+	+	+
CR2	+	+	+
CR3	+	+	-
CR4	+	+	+

Heavy Metal Resistance of the Bacterial Isolates

The results revealed that the tolerance level of CR1 (*A. viridans*) for the all concentration (mg/L) values of lead (Pb) does not vary significantly ($p<0.05$). The highest zone of inhibition of 7.44±1.74 mm was recorded in 1.0mg/L concentration of chromium salt while the lowest zone of inhibition (2.50±1.26 mm) was recorded in 0.05mg/L concentration of lead salt. The tolerance of *A. viridans* to chromium and manganese showed significant difference ($p<0.05$) across all the concentration levels of the metal salts (Table 6a).

The mean value concentration of heavy metal tolerance of CR2 (*Peribacillus simplex*) as presented in Table 6b, showed that the concentrations of lead (Pb) does not vary significantly ($p<0.05$). Chromium recorded the highest zone of inhibition (14.29±3.38 mm) at 1.0mg/L concentration while the lowest zone of inhibition of 4.14±1.44 mm was observed in 0.05mg/L concentration of both lead and manganese salts. The tolerance of *Peribacillus simplex* to chromium and manganese showed significant difference ($p<0.05$) across all the concentration levels (Table 6b).

As depicted in Table 6c, the heavy metal tolerance of the CR3 (*Pseudomonas aeruginosa*) revealed that the concentration (mg/L) values of lead (Pb) does not vary significantly ($p<0.05$). High zones of inhibition were observed in nickel, manganese and chromium (5.00±2.55 mm, 6.12±2.48 mm and 9.34±1.64 mm respectively) against CR3 at 1.0ml/L concentration level, and also varied significantly across the other concentrations level.

The Mean values concentration of heavy metals tolerance of the CR4 (*Staphylococcus epidermis*) as depicted in Table 6d, showed that the concentration (mg/L) values of Lead (Pb) and Nickel (Ni) do not show any significant difference ($p<0.05$) whereas chromium and manganese showed significant difference ($p<0.05$) across all the concentration levels. *Staphylococcus epidermis* recorded the highest zone of inhibition (17.54±3.38 mm) in 1.0mg/L concentration of chromium salt and the lowest zone of inhibition (2.12±1.64 mm) was observed in 0.05mg/L concentration of lead salt (Table 6d).

Table 6a: Heavy Metals Tolerance of CR1 (*Aerococcus viridians*)

Heavy Metal Concentration (mg/L)	**Zone of inhibition(mm) (Mean values from day 1- day 7 of incubation)**			
	Lead	**Nickel**	**Manganese**	**Chromium**
1.00	4.10±2.69^{a}	5.20±1.73^{b}	6.40±3.45^{b}	7.44±1.74^{b}
0.25	3.12±2.69^{a}	3.00±1.09^{a}	4.43±1.16^{a}	5.53±1.21^{a}
0.15	3.00±1.38^{a}	2.77±1.90^{a}	4.34±1.56^{a}	4.83±1.83^{a}
0.05	2.50±1.26^{a}	2.67±1.29^{a}	3.24±1.64^{a}	4.74±1.54^{a}

Data is expressed as mean ± Standard Error; Different alphabets along the column indicate significance difference ($P>0.05$) using Duncan Multiple Range Test (DMRT).

Table 6b: Heavy Metals Tolerance of CR2 (*Peribacillus simplex*)

Heavy metal Concentration (mg/L)	(Zone of inhibition(mm) (Mean values from day 1- day 7 of incubation)			
	Lead	**Nickel**	**Manganese**	**Chromium**
1.00	6.14±1.64[a]	9.00±2.43[b]	10.00±2.55[b]	14.29±3.38[b]
0.25	5.43±1.53[a]	7.57±2.08[a]	8.29±1.06[a]	12.00±2.49[ab]
0.15	4.43±1.51[a]	5.57±1.80[a]	6.14±1.06[a]	9.00±2.14[a]
0.05	4.14±1.44[a]	4.43±1.59[a]	4.14±1.06[a]	8.00±1.76[a]

Data is expressed as mean ± Standard Error; Different alphabets along the column indicate significance difference (P>0.05) using Duncan Multiple Range Test (DMRT).

Table 6c: Heavy metals tolerance of CR3 (*Pseudomonas aeruginosa*)

Heavy metal concentrations (mg/L)	Zone of inhibition(mm) (Mean values from day 1- day 7 of incubation)			
	Lead	**Nickel**	**Manganese**	**Chromium**
1.00	3.89±2.43[a]	5.00±2.55[b]	6.12±2.48[b]	9.34±1.64[b]
0.25	3.10±2.08[a]	4.34±1.06[a]	4.10±2.69[ab]	7.98±1.53[ab]
0.15	2.00±2.80[a]	3.69±2.06[a]	3.00±1.38[ab]	5.78±1.51[a]
0.05	1.78±1.56[a]	2.24±1.06[a]	2.50±1.26[a]	5.24±1.44[a]

Data is expressed as mean ± Standard Error; Different alphabets along the column indicate significance difference (P>0.05) using Duncan Multiple Range Test (DMRT).

Table 6d: Heavy metals tolerance of CR4 (*Staphylococcus epidermis*)

Heavy Metal Concentration (mg/L)	Zone of inhibition(mm) (Mean values from day 1- day 7 of incubation)			
	Lead	**Nickel**	**Manganese**	**Chromium**
1.00	4.23±1.64[a]	6.30±1.64[a]	9.00±2.55[b]	17.54±3.38[b]
0.25	3.43±1.53[a]	5.56±1.53[a]	5.29±1.06[a]	11.20±2.69[ab]
0.15	2.94±1.51[a]	4.94±1.51[a]	4.83±1.61[a]	9.53±2.21[ab]
0.05	2.12±1.64[a]	4.78±1.64[a]	4.54±2.16[a]	6.20±1.01[a]

Data is expressed as mean ± Standard Error; Different alphabets along the column indicate significance difference (P>0.05) using Duncan Multiple Range Test (DMRT).

DISCUSSION

The spatial distribution of endophytic organisms usually depends on seasonal variation, rainfall, soil parameter, location of plants, plant age and genotype (Lamit *et al*., 2014). In this study, four endophytic bacteria strains were isolated from *Chamaecrista rotundifolia* growing on heavy metal-contaminated Nigerian Air Force shooting range, Kaduna. On the basis of morphological identification, biochemical characterization and 16S rRNA gene sequence, the endophytic bacterial strains isolated from the roots of *C. rotundifolia* were identified as *Aerococcus viridans, Peribacillus simplex, Stapylococcus epidermis* and *Pseudomonas aeruginosa.* The result of this study is similar to that of Ma *et al*. (2009), Rashid *et al*. (2012) and Liu *et al*. (2015) who reported these bacterial species as endophytes of many plants growing on heavy metal-contaminated site.

Verma *et al.* (2001) reported that metal tolerance holds an association with antibiotic resistance. A similar kind of multiple antibiotic resistance property was observed from the four isolates in this study where the isolates showed great resistance to most of the antibiotics. Resistance to perfloxacin (5µg), Septrin (30µg), Nalidixic acid (10µg), Amoxacillin (30µg), Gentamycin (10 µg), Ampiclox (30µg), (30µg) and Streptomycin (25µg), Erythromycin (15µg), Zithromax (10ug) but sensitive(S) to ciprofloxacin (10 µg) was observed in *Pseudomonas aeruginosa* which clearly indicates that the high degree of antibiotic resistance might be associated with higher levels of tolerance to various heavy metals (Zhang *et al*., 2014). Rogers *et al.* (2004) reported that the transfer of antibiotic resistance gene from one bacterium to another is a reason for the high antibiotic resistance pattern in some bacteria. Under conditions of imposed stress, metal and antibiotic resistance in microorganisms possibly helps them to adopt spontaneously than by mutation and natural selection (Zhang *et al*., 2014). *Aerococcus viridans, Peribacillus simplex* and *Staphylococcus epidermis* being from the same phylum Firmicutes, have also been reported to be resistant to antibiotics due to their very strong cell wall. *Aerococcus viridans* is highly susceptible to penicillin in a lot of clinical samples (Rogers *et al.,* 2004).

The Plant Growth Promoting Properties of isolated endophytic bacteria indicate that they have high potential to be used as biofertilizers and microbe-assisted phytoremediators (Zhang *et al.,* 2011). Hontzeas *et al.* (2004) stated that IAA were capable of stimulating plant biomass and the ACC deaminase has been proposed to play a key role in microbe-plant association.

Aerococcus viridans and *Pseudomonas aeruginosa* recorded relatively high tolerance and minimal zone of inhibition from day one to day seven of incubation of the study compared to the two other isolated endophytic bacterial strains, *Peribacillus simplex* and *Staphylococcus epidermis*. In the study carried out by Maitra (2016) similarly revealed that *Pseudomonas aeruginosa* has the ability to tolerate heavy metals. Pal *et al.* (2004) stated that three strains of *P. aeruginosa* (S6, S7 and S8) isolated from natural polluted environments on nutrient agar were tolerant to $CuSO_4$, $Cr_2(SO_4)_3$, $CoSO_4$ corresponding to 1.6, 1.2 and 0.8mM, respectively, of metal ions concentrations. Also, Raja *et al*. (2006) reported that *P. aeruginosa* showed tolerance up to concentration 3.8,4.4, 7.6 and 11.9mM on LB medium to Pb^{2+}, Cd^{2+}, Cr^{6+} and Ni^{2+} respectively. Chen *et al.* (2005) have previously reported that reduction in growth is mainly because of the interaction between the cell surface and of metal cations along with phosphate, carboxyl, hydroxyl and amino-groups. Although lead (Pb) is known for its toxicity as documented by Manton *et al*. (2000), it was observed that the endophytic bacteria in this study thrived well in the presence of lead; this is also in agreement with Elizabeth *et al*. (2017) who in their study, reported that endophytic bacteria grow well in the presence of lead. Efficient heavy metal-removing capabilities and the ability to grow over a wide range of metal concentrations under aerobic conditions along with antibiotic resistance are clear indications of the advantages that may offer to employ this organism for metal remediation in simple reactors or *in situ* (Zhang *et al*., 2014). Phytoremediation with endophytic microbes is an important new means of improving bioremediation efficacy (Andreolli *et al.*, 2013; Zhang *et al*., 2014).

CONCLUSION

Endophytic bacteria that inhabited the root nodules of *Chamaecrista rotundifolia* growing on the Nigerian Air Force shooting range, Kaduna, were isolated and molecularly identified as *Aerococcus viridans*, *Peribacillus simplex*, *Pseudomonas aeruginosa and Staphylococcus epidermis.* The possession of antibiotic resistance, plant growth promoting properties and heavy metal resistance by these endophytic bacterial isolates, indicate that they could exhibited greater potential for improving phytoremediation of heavy metal-contaminated sites. The bioavailability of metals to plant roots is considered to be a critical requirement for plant metal bioconcentration or bio-immobilization to occur. Therefore, it may be possible to employ beneficial bacteria to alter the bioavailability of metals for improving phytoremediation of metal contaminants on large scale in the environment. This study recommends a deeper understanding of the interaction between the isolated endophytic bacteria and their plant host *Chamaecrista rotundifolia*, through the functional genomics analysis of the endophyte; this understanding will benefit the application of endophytic bacteria to phytoremediation.

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