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Molecular characterization and evaluation of crude oil remediation potential of some rhizobia isolated from plant root nodules

Jesupemi Mercy Enibukun✉ and Bolatito Esther Boboye

Department of Microbiology, Federal University of Technology, PMB 704, Akure, Ondo state, Nigeria

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Abstract

This study aimed to determine the molecular identities and genetic relatedness of rhizobia isolated from pigeon pea and pinto beans, and assess their remediation potential in the presence of 1 %, 3 % and 5 % (w/v) crude oil in minimal medium for 7 days incubation period. Standard microbiological and molecular methods which include amplification and purification of 16S rRNA, agarose gel electrophoresis, and sequencing. Results showed molecular identities of six rhizobia from pigeon peas as *Bradyrhizobium diazoefficiens* USDA122, *Rhizobium leguminosarum* WSM2304, *B. japonicum* N61, *R. leguminosarum* N741, *R. leguminosarum* BIHIB1217, and *B. japonicum* E109; and three rhizobia obtained from pinto beans were *R. leguminosarum* N871, *B. diazoefficiens* USDA110 and *B. japonicum* SEMIA5079. All tested rhizobia (9) showed petroleum degradation ability, as they all grew in the 1, 3 and 5 % (w/v) crude oil minimal medium under laboratory conditions. *B. diazoefficiens* USDA122 showed the highest optical density (OD) value of 1.184 ± 0.05 on 7th day at 1 % (w/v) crude oil contamination, while *R. leguminosarum* N741 has the lowest OD value of 0.372 ± 0.02 at 5 % (w/v) crude oil on 7th day. For all the rhizobia, increase occurred throughout incubation period at 1, 3 and 5 % (w/v) except *R. leguminosarum* N741 and *R. leguminosarum* BIHIB1217. In conclusion, the association of *R. leguminosarum* BIHIB1217 and *R. leguminosarum* N871 from pigeon pea and pinto beans respectively, were found most effective in crude oil degradation and thus they are recommended as a promising association for remediation of crude oil spilled soils.

Introduction

The ability of humans to change the environment has increased faster than the ability to predict the effect of that change (Ikuesan 2015). Pollution of the environment is one of the major effects of human technological advancement. Pollution can be defined as the introduction of deleterious substance into the environment that endangers human health and other natural resources (Onojake 2004). It results when a change in the environment harmfully affects the quality of human life and other essential living things such on animals, microorganisms and plants (Ogbogbodo *et al.* 2005; Eremrena and Akonye 2013).

Crude oil is a naturally occurring complex mixture of thousands of hydrocarbons and non-hydrocarbon compounds, including heavy metals. Organic chemicals such as hydrocarbons are major constituents of petroleum, which can enter the aquatic environment through natural and anthropogenic sources (Daniel and Nna 2016). Total petroleum hydrocarbon (PHC) is a mixture of measurable number of petroleum-based

✉ *Corresponding author:* jesupemienibukun@gmail.com

hydrocarbons found in crude oil in an environmental media (Rauckyte *et al.* 2010). Some of the chemicals found in TPH are hexane, benzene, toluene, xylene, naphthalene as well as other petroleum products and gasoline components. Crude oil contamination constitutes one of the most prevalent sources of environmental pollution due to variation in chemical composition of crude oil and its products degradation in the industrialized world (Mandal *et al.* 2012). Crude oil pollution can be defined as the introduction of crude oil or its derivatives with its associated gases into the environment (air, water and land) in quantities that are poisonous or capable of causing immediate physical, chemical and biological damages to the affected ecosystem (Lee *et al.* 2001; Inoni 2006; Tanee and Anyanwu 2007; Nyananyo 2008; Tanee and Akonye 2009). Petroleum industry's effluents, oily sludge and oil spills cause a serious threat to the environment as their constituents are toxic, mutagenic and carcinogenic (Nyananyo 2008; Wokoma 2014; Ataikiru *et al.* 2018).

The remediation of oil contaminated soils has been a major problem in oil producing countries and recently use of plants to clean such soils has been investigated. Several methods such as physical and chemical means have been adopted in remediation of hydrocarbon polluted soil or the detoxification of hazardous substances (Diplock *et al.* 2009). However, these methods were not effective in the total recovery of polluted soils. Phytoremediation and bioaugmentation engage the process of converting organic waste into an innocuous state by the use of plants and microbes respectively. Due to the abilities of certain microbes to mineralize hydrocarbon components into environmentally friendly substances such as carbon dioxide and water, the ability of bacteria in breaking down hydrocarbons has gained growing attention in modern day research (Kadali *et al.* 2012; Gkorezis *et al.* 2016). The degradative activity of bacteria isolated from hydrocarbon polluted soil has been investigated by monitoring the changes in optical density (OD) which was directly proportional to the activity (Boboye *et al.* 2010). There is premise that the genome of these organisms, harbour genes or enzymes responsible for the degradation (Gkorezis *et al.* 2016).

Legumes belong to the family *Fabaceae* or *Leguminosae*. Legumes are plants that form symbiotic association with rhizobia in the symbiosis legumes nitrogen fixing root or stem nodules. Legumes include cowpea, soybean, pigeon peas, pinto beans, alfalfa, chicken peas, lentils, peanut and lupin beans (Ismail *et al.* 2014). *Cajanus cajan* (L.) which commonly called pigeon pea, red gram, congo pea, or gungo pea, is one of the most common legumes cultivated in the tropics and subtropics, for its edible seed (Fig. 1). It is a legume domesticated majorly in the India

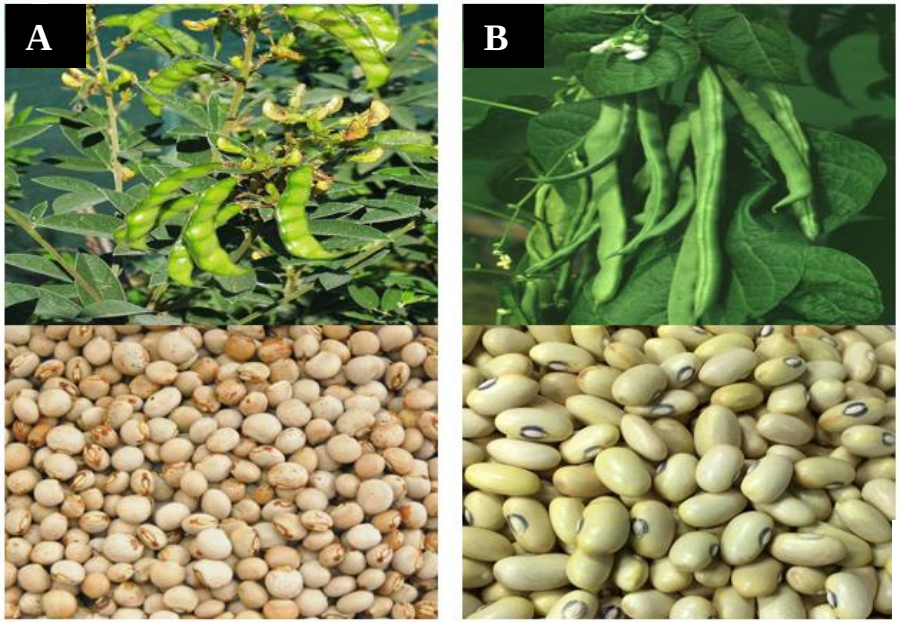

Fig. 1. The plant and seed of (**A**) pigeon pea (*Cajanus cajan*) and (**B**) pinto beans (*Phaseolus vulgaris*).

and North-eastern Africa in over 3,000 years ago (Mallikarjuna *et al.* 2011). Pigeon pea is found useful in many areas as growth enhancer, alley crop, and protein and trace nutrient supplement, control of weeds and nematodes as well as medication (Odeny 2007). The pinto beans (*Phaseolus vulgaris*) are legumes referred to as common beans (Fig. 1), they spread throughout South and Central America, Europe, Africa and Asia (Rodino *et al.* 2001). Pinto beans are the most highly consumed dried bean in the United States. Its protein content is comparable with those in other legumes like cowpea and groundnut which had been used in complementing maize diet rich in mineral and fibre.

Legumes are known to have an advantage over non-leguminous plants in phytoremediation because of their ability to fix nitrogen and thus, do not have to compete with microorganisms and other plants for limited supplies of available soil nitrogen at oil-contaminated sites (Cameron 2003). The root nodule symbiosis established between legumes and rhizobia is an exquisite biological interaction responsible for fixing a significant amount of nitrogen in terrestrial ecosystems. The success of this interaction depends on the recognition of the right partner by the plant within the richest microbial ecosystems on earth, the soil. The economic and ecological importance of legumes is evidenced by the high number of species that are cultivated and commercialized, as well as by their ability to obtain nitrogen from a symbiotic interaction with soil bacteria known as rhizobia (Oberai and Khanna 2018).

Plant–microbial interactions will be better understood by considering the organism in its natural environment together with its microbiome (Thijs *et al.* 2016). 16S rRNA gene sequencing is a well-utilized method for nucleic acid-based detection and identification of microbes, their taxonomic assignment, phylogenetic analysis and the study of microbial diversity (Quast *et al.* 2013). The objective of this study was to isolate and determine the molecular identities and phylogenetics of some tropical rhizobia from pigeon pea and pinto beans, and assess the crude oil degradative ability of these isolated rhizobia. Since nitrogen act as alternative to chemical fertilizers, the study of the crude oil bioremediation potential of rhizobia-legume association will provide strategy for farming on polluted agricultural soil.

Experimental

Source of legumes

Pigeon pea (*Cajanus cajanus*) and pinto beans (*Phaseolus vulgaris*) legumes were sourced from Oba market, Akure, Nigeria. The legumes were identified and authenticated at the Department of Crop, Soil and Pest management (CSP) of the Federal University of Technology, Akure, Nigeria.

Materials, media and reagents

All reagents used in this study were of analytical and microbiological standard. Nutrient agar (NA) composed of: meat extract (1 $g.L^{-1}$); peptone (5 $g.L^{-1}$); yeast extract (2 $g.L^{-1}$); sodium chloride (8 $g.L^{-1}$); agar (15 $g.L^{-1}$); and distilled water (1 L), at pH 6.8. Mineral Salt Medium (MSM) composed of ($g.L^{-1}$): 1 g NaCl, 1 g KH_2PO_4, 1 g Na_2HPO_4, 0.5 g NH_4NH_3, 0.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 0.02 g $CaCl_2.2H_2O$, 0.002 g $FeCl_3$, and 0.002 g $MnSO_4.2H_2O$ in 1.0 litre of distilled water, at pH 7.0 and sterilizes at 121 ^oC for 15 min by autoclave. Modified Mineral Salt Medium (MMSM) consisted of ($g.L^{-1}$): 4 g KH_2PO_4, 4 g NH_4NH_3, 0.2 g $MgSO_4.7H_2O$, and 0.01 g $CaCl_2.2H_2O$, in 1.0 L of distilled water, at pH 7.0 and sterilized at 121 ^oC for 15 min by autoclave. MSM was used for growth of isolated mutants that is used in this experiment while MMSM was used to encourage oil emulsion process which leads to oil degradation.

Isolation of Rhizobium *from legumes and cultivation of test bacteria*

Surface sterilization of the seeds were done with 3.5 % (w/v) sodium hypochlorite solution for 15 min and rinsed in sterile distilled water. The seeds were transferred into sterile sandy soil in different sterilized plant jar, regularly wet with MMSM and allowed to germinate at 28 ± 2 ^oC (daytime) for 90 days in a locally-made greenhouse facility. The root nodules of the germinated plants were used respectively. The root aggregates were

collected and sterilized using 3.5 % (v/v) hypochlorite solution, sterile root was crushed with tweezers, then the root nodule extracts were grown on nutrient agar, incubated at room temperature (28 ^{o}C) for 24 hours. Bacterial colonies were gram stained by method of Fawole and Oso (2007); the bacterial cells that retained the purple stain from the crystal-violet staining were referred to as Gram positive and those that showed pink from the safranin staining were referred to as Gram negative. Each was cultured in nutrient broth overnight at 30 ^{o}C until log phase (optical density at 600 nm) and used for molecular characterization.

Identification and molecular characterization of bacterial isolates

Total DNA was extracted from 2 mL of each grown bacterium. The cells were pelleted by centrifuging at 12,000 rpm for 2 min. The pellet was mixed with TRIS-EDTA (TE) buffer and treated with sodium dodecyl sulphate for 10 min. Isopropanol (600 µL) was added and centrifuged at 10,000 rpm for 10 min and decanted. The pellet formed was dissolved in 0.1 mL of a TE buffer. The DNA was quantified using Nanodrop machine at 260 nm and 280 nm. The 16S rRNA gene was amplified using primers: (i) 5' AGAGTTTGATCCTGGCTCAG 3' and (ii) 5' GACGGGCRGTGWGTRCA 3'. The polymerase chain reaction (PCR) mix contained 10X buffer, 100 mM dNTPs, 2.52 M $MgCl_2$, 2U *Taq* DNA polymerase, 1.0 µL of each primers, 2.0 µL of the extracted DNA and sterilized distilled water to make a final volume of 25 mL. The mixture was subjected to amplification on PCR machine with the following parameters: initial 94 °C 3 min, followed by 25 cycles run in a thermal cycler, each comprising of 1 min at 94 °C, 1 min at 94 °C and 1.5 min at 94 °C. The final denaturation was at 94 °C for 10 min. About 2.0 µL of the PCR product was subjected to 1.0 % (w/v) agarose-gel electrophoresis at 90 V for about 30 min, and the resulted gel was observed under UV transilluminator and photographed. 1 µL of the amplified DNA was sequenced on the Applied Biosystems Genetic Analyzer at facility in International Institute of Tropical Agriculture (IITA), Ibadan Nigeria. The DNA sequence of the PCR product of the 16S rRNA gene of each bacterium was subjected to homology analysis using BLASTN program of the National Center for Biotechnology Information (NCBI).

Preparation of bacterial inoculum

Nine rhizobia that grew equal to and above 0.600 optical density in the presence of crude oil at the end of the 7 days evaluation experiment on crude oil degradative capability were used. The rhizobia were inoculated into 5 mL nutrient broth and incubated at 28 ^{o}C for 24 h. It was centrifuged at 4,000 rpm for 5 min. The cells were re-suspended in 5 mL sterile distilled water. This constituted the bacterial inoculum.

Evaluation of crude oil degradative capability of the test bacteria

The mineral salt medium (Bushnell-Hass) was prepared. The medium was allowed to cool and crude oil was sterilized using 0.45 µm Millipore filter and added to make final crude oil concentration of 1 %, 3 % and 5 % volumes of 1, 3 and 5 mL. Uninoculated MSM broth containing the crude oil served as the control. The broth was inoculated with each *Rhizobium* species and incubated at 28 ± 2 °C for 7 days. Culture tubes were agitated at 4,000 rpm daily using shaking incubator to provide adequate oxygen for the bacteria to grow. During the incubation the optical density of each cultured rhizobium was read daily with spectrophotometer at 600 nm (Boboye *et al*. 2010).

Statistical Analysis

The data were taken in triplicates and expressed as mean ± standard deviation (SD) using Microsoft Excel.

Results

The concentration of DNA from rhizobia samples ranged between 186.6 to 277.6 $ng.\mu L^{-1}$. The 260/280 ranged between 1.81 to 1.97, and indicated the quality of DNA of all the rhizobia were good. The 16S rRNA fragment was amplified by PCR on DNA from rhizobia. Gel separation

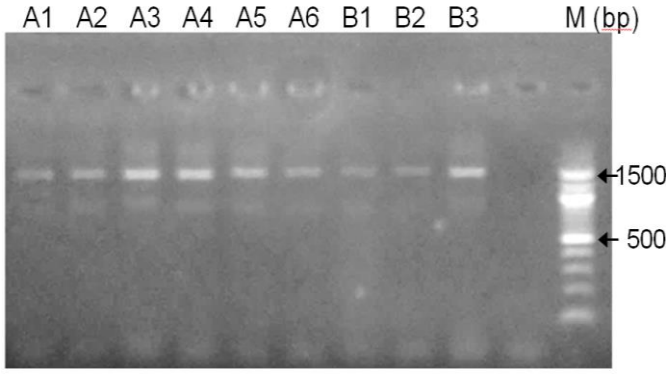

Fig. 2. The PCR products amplified on 16S rRNA gene of rhizobia isolated from pigeon pea (isolates A1-A6) and pinto bean (isolates B1-B3). Lane M represents the DNA ladder.

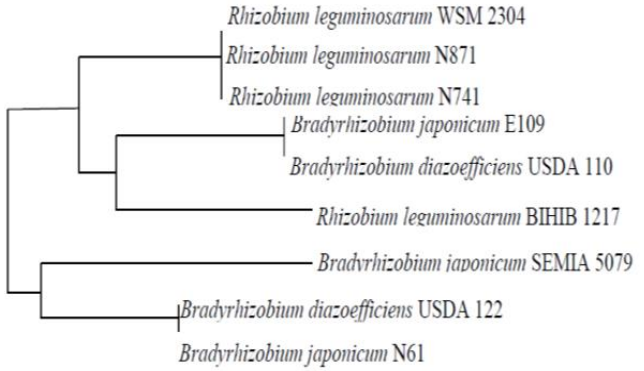

Fig. 3. A phylogenetic tree of the crude oil degrading rhizobia identified by molecular analysis.

confirmed the presence of a 1500 bp fragment (Fig. 2). Arrangement of the nucleotides in the PCR product from the DNA extracted from each of the rhizobia isolated from pigeon pea and pinto beans. The number of each base in the total nucleotides in each rhizobium varied from one test bacterium to the other with total nucleotides ranging from A1 to A6 (403 to 551) bases for bacterium isolated from pigeon pea. The molecular identities of the bacteria isolated from pigeon pea and pinto beans are shown in Table 1. Among the bacteria isolated from pigeon pea were three species *Bradyrhizobium* and three species of *Rhizobium.* In pigeon pea, the percentage identity was lowest for isolate coded A6 (96 %) and highest (100 %) for bacteria A1, A, A3, and A5. The molecular identities of the bacteria associated with pinto beans were two species of *Bradyrhizobium* and one species of *Rhizobium*. The percentage identity for the bacteria isolated was the lowest for isolate B2 (99 %) and highest (100 %) for B1 and B3.

The phylogenetic tree (Fig. 3) shows the relatedness of the bacteria isolated from pigeon pea and pinto beans. The phylogenetic tree of the isolate has three clades. From the first clade, there are three strains of *Rhizobium leguminisarum* (WSM2304, N871, and N741) which are closely related. In the second clade *Bradyrhizobium japonicum* E109 is closely related to *Bradyrhizobium diazoefficiens* USDA 110 than *Rhizobium leguminisarum* BIHB 1217 but shares the same ancestors. *Bradyrhizobium diazoefficiens* USDA 122 and *Bradyrhizobium japonicum* SEMIA5079 are closely related but shares a distance relationship with the other identified rhizobia *Bradyrhizobium japonicum* N61 in the third clade.

We studied the ability of the test rhizobia to grow in the presence of crude oil, supplied to growth media at concentrations 1 %, 3 % and 5 % (v/v). The OD values were taken as measure of growth thus crude oil degradation. Our data showed that crude oil differently affected the growth of individual isolates from pigeon bean (Fig. 4) as well as from pinto beans (Fig. 5). Generally,

Table 1. Molecular identities of bacteria associated with pigeon pea and pinto beans.

Rhizobia code	Identified bacteria	Identity [%]	Accession number
A1	*Bradyrhizobium diazoefficiens* USDA122	100	CP013127.1
A2	*Rhizobium leguminosarum* WSM2304	100	CP01193.1
A3	*Bradyrhizobium japonicum* N61	100	CP017637.1
A4	*Rhizobium leguminisarum* N741	99	CP013595.1
A5	*Rhizobium leguminosarum* BIHIB 1217	100	CP022665.1
A6	*Bradyrhizobium japonicum* E109	96	CP010313.1
B1	*Rhizobium leguminisarum* N871	100	CP013590.1
B2	*Bradyrhizobium diazoefficiens* USDA 110	99	CP011360.1
B3	*Bradyrhizobium japonicum* SEMIA5079	100	CP007569.1

A1-A6 represent bacteria isolate from pigeon pea. B1-B3 represent bacteria isolate from pinto beans

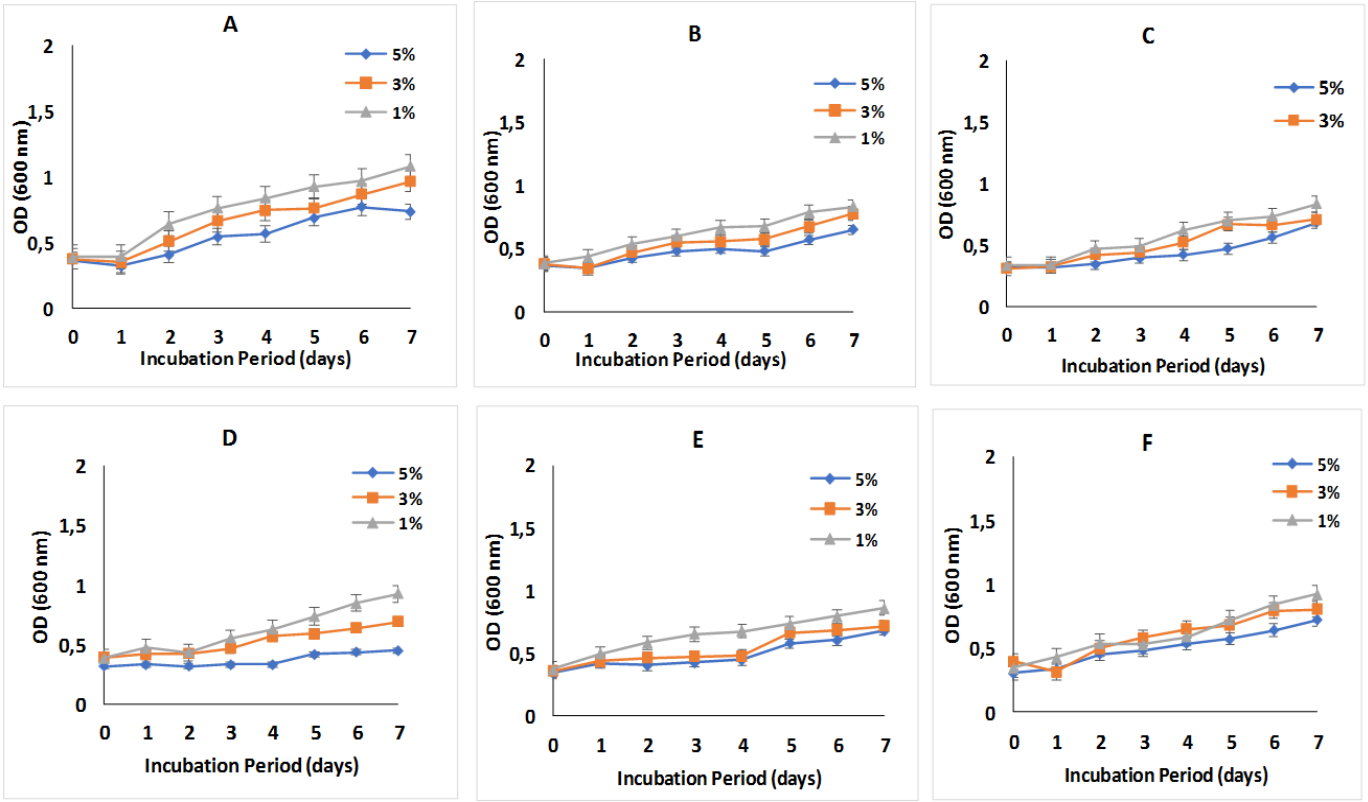

Fig. 4. Growth of test rhizobia isolated from pigeon pea in presence of crude oil in the medium (1 – 5 % w/v). Data represent means ± SD (n = 3). **A** = *B. diazoefficiens* USDA122; **B** = *R. leguminosarum* WSM2304; **C** = *B. japonicum* N61; **D** = *R. leguminosarum* N741; **E** = *R. leguminosarum* BIHIB1217; **F** = *B. japonicum* E109.

addition of 1 % (w/v) crude oil promoted rhizobia growth the most, followed by 3 % and 5 % oil concentration (Fig. 4 and 5). The effect of oil was significant at $P < 0.05$. Best growth was observed for *B. diazoefficiens* USDA122 at 1 % (w/v) crude oil contamination (OD 1.184 ± 0.05 on 7th day). In contrast, most inhibited was the growth of *R. leguminosarum* N741 (OD 0.372 ± 0.02 on 7th day) by 5 % (w/v) crude oil in growth media. Relatively flat growth curve was obtained for the rhizobia isolate *B. diazoefficiens* USDA122 (Fig. 4A). Of the rhizobia isolated from pigeon pea plants, *B. japonicum* E109 had the highest growth rate (OD 0.899 ± 0.06 on 7th day), while the lowest one was obtained for *B. diazoefficiens* USDA122 on day 6 (Fig. 4A and F).

Moreover, among the rhizobia isolated from pinto beans plants, *B. japonicum* SEMIA5079 had the highest OD (1.107 ± 0.05 on 7th day) when compared to the OD obtained for *R. leguminosarum* N871 (Fig. 5A and C). The increase in optical density of each test bacteria in the presence of crude oil indicates utilization of the oil for growth and degradation of the test hydrocarbon. The results of this study showed that each test bacteria could survive and utilize crude oil at the percentages investigated.

Discussion

In bacteria, 16S rDNA forms core conserved region with about 1,500 base pair. The region is employed for bacteria identification. This correlated with data obtained by Mohamed *et al.* (2014) that 16s rRNA region is involved in bacterial identification.

Studies have shown that endophytic bacteria have a better capacity to enhance petroleum hydrocarbon (PHC) phytoremediation than rhizosphere or soil bacteria (Weyens *et al.* 2010; Yousaf *et al.* 2011). Cultivable endophytic bacteria have been isolated from various plants species which include maize (Gutierrez-Zamora and Martinez-Romero 2001), wheat (Larran *et al.* 2002), sugar cane (Loiret *et al.* 2004), and *Mimosa pudica* (Pandey *et al.* 2005).

A molecular characterization study on endophytic

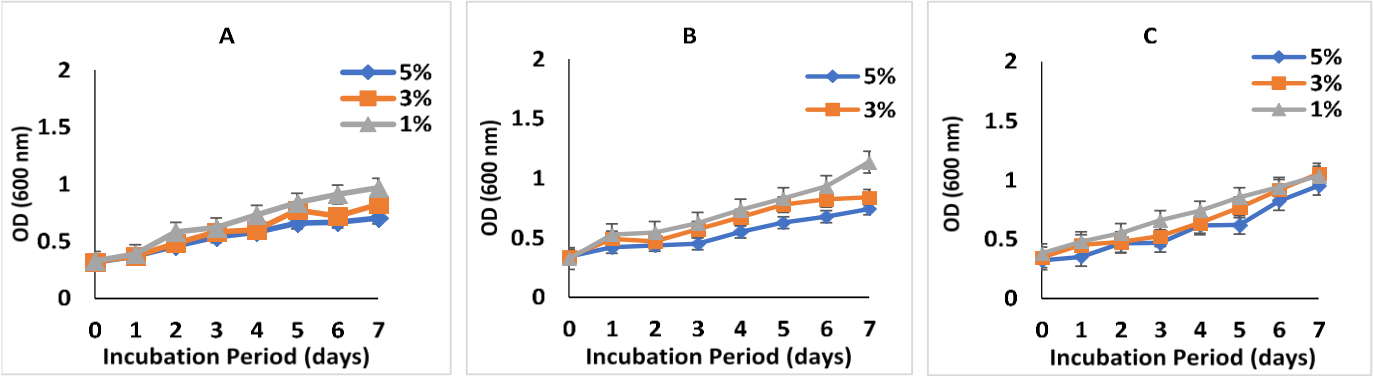

Fig. 5. Growth of test rhizobia isolated from pinto beans in presence of crude oil in the medium (1 – 5 % v/v). Data represent means ± SD (n = 3). **A** = *R. leguminosarum* N871; **B** = *B. diazoefficiens* USDA110; **C** = *B. japonicum* SEMIA5079.

bacteria isolated from leguminous plants nodules and roots cultivated in the rhizosphere soil of *Acacia* reported the presence of *Ensifer meliloti* (*Sinorhizobium meliloti*) in nodules of chickpea and common bean as 40.9 %, *Rhizobium* sp. in nodules and roots of common bean and lentil as 31.8 %, and *Enterobacter* sp. isolated in roots of faba bean, common bean, chickpea and lentil as 27.3 % (Taoufiq *et al.* 2018).

The increasing in optical density of each test bacteria in the presence of crude oil indicates the survival and utilization of the oil for growth and degradation of the test hydrocarbon. The bacteria have strains that utilized the crude oil as a sole source of carbon and energy since there was no other source of carbon and energy. This *Rhizobium* responded to crude oil and showed increased optical density at the end of the seventh day. This means that the genes encoding each rhizobium were not inhibited by the crude oil hydrocarbons. These microorganisms are likely to possess enzymatic capacity to degrade the crude oil (Olukunle and Boboye 2013). Thus, increase in the hydrocarbon utilizing bacterial population conforms to the data from the work done by Lawson *et al*. (2012) and Ataikiru *et al*. (2018).

Lawson *et al.* (2012) have reported that introduction of PHC into the soil encourages high microbial biomass. Joshi and Pandey (2011) also suggested that the percentage of petroleum utilizing bacteria in a particular environment appears to be an index of the presence of hydrocarbons in the environment. The bacteria might have broken the oil down into simple carbon compounds that are used to make the sugars, fat and proteins needed for growth and energy production ultimately the by-product become carbon dioxide and water (Okerentugba and Ezeronye 2003). This finding agrees with the report of Salam *et al*. (2011) that both Gram-negative and positive bacteria have been implicated in the mineralization of hydrocarbon pollutant. The dominance of Gram-negative bacteria in the samples agrees with that of Kaplan and Kilts (2004), that Gram-positive bacteria, when detected in bioremediation, are never diverse or dominant. The high moisture content of the oils might have provided the moisture necessary for bioactivity thus supporting the growth and survival of these microbes.

Reduction in OD of the oil medium at different incubation times could be due to possible disappearance of the crude oil in the medium due to exhaustion of the hydrocarbon which served as the carbon source for various metabolic activities. The difference in oil degradation ability of the rhizobia could be attributed to the difference in their genetic abilities in utilizing hydrocarbons as substrate vis-à-vis absorption, oxidation and environmental factors. Similar suggestion was made by Majid *et al*. (2008) and Onuoha *et al*. (2014) when they studied spent oil degradation with some bacteria. Rhizobia used in this research could play a vital role in plant nutrient cycling and replenishment in the soil. These microbes are also important for hydrocarbon degradation in soil. Their role includes the degradation and biotransformation of petroleum compounds into simple harmless compounds (Oluyege *et al.* 2011).

Biodegradation by microbes is the key removal process of hydrocarbons which is controlled by physicochemistry, environmental conditions,

bioavailability and the presence of catabolically active microbes (Stroud *et al.* 2007). One of the major challenges with phytoremediation is that it takes long time. Large-scale clean-up approach was applied to tackle the Deepwater Horizon oil spill in the USA, by direct delivery of a dispersant that boosted the natural biodegradation of the oil (Atlas and Hazen 2011). Also, studies have shown that a field-scale process of contaminated soil bioremediation is possible (Pelaez *et al.* 2013; Pizarro-Tobías *et al.* 2015).

Conclusion

In conclusion, this work has impacted the understanding and effectiveness of the crude oil degradative abilities of the isolated and tropical rhizobia species using their compactible legumes to degrade crude oil in soil. The use of plant-associated bacteria such as rhizobia to degrade toxic synthetic organic compounds in environment may provide an efficient, economic and sustainable bioremediation technology. This laboratory scale research study can also be applied on a large-scale study thus, are environmentally friendly and have been observed to promote the biodegradation of hydrocarbons. The results of this study showed that each test bacteria could survive and utilize crude oil at the percentages investigated. Thus, they will be useful to fixed nitrogen when added to (bio-augmentation and bio-stimulation strategies) the root of the pigeon peas and pinto beans planted in a crude oil polluted soil.

Conflict of Interest

The authors declare that they have no conflict of interest.

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