

Characterization of Hydrocarbon-Utilizing Bacteria Associated with Kenaf (*Hibiscus cannabinus* L.) Plant Grown in a Niger Delta Soil

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Research Article

Abstract

Hydrocarbon Utilizing Bacteria (HUB) were isolated at the juvenile and maturity stages from the core and rhizosphere of Tianung 1, Tianung 2 and Cuba 108 accessions of Kenaf (*Hibiscus cannabinus*) plant grown in a hydrocarbon unimpacted soil in the Niger Delta Area. Standard culture dependent techniques were used for the enumeration of heterotrophic and hydrocarbon-utilizing bacteria while PCR amplification of a fragment of the 16S rRNA bacterial gene and sequencing was done to identify the HUB isolates to species level. Sequences obtained were deposited at the Gen Bank of the National Centre for Biotechnology Information (NCBI) and accession numbers were obtained. The rhizospheric heterotrophic bacterial counts between plants 1 and 2 of the various accessions ranged from 1.41×10^7 to 2.4×10^8 cfu/g at the juvenile stage and 1.44×10^7 to 6.5×10^8 at maturity, while rhizospheric hydrocarbon utilizing bacterial counts ranged from 3.0×10^7 to 4.7×10^7 (cfu/g) (juvenile stage) and 2.7×10^7 to 5.0×10^7 (cfu/g) (maturity). One way Analysis of Variance showed significant difference ($P < 0.05$) only between hydrocarbon utilizing bacterial counts and total heterotrophic bacterial counts for plant 1 only. Thirteen (13) HUB species were identified: *Providencia vermicola* A10-01, *Providencia rettgeri* B10_04, *Providencia rettgeri* D04-10, *Pseudomonas aeruginosa* D10-10, *Providencia vermicola* F10-16, *Providencia rustigianii* H10-22, *Lysinibacillus fusiformis* B11-05, *Lysinibacillus sphaericus* C11-08, *Exiguobacterium aurantiacum* F03-18 *Klebsiella pneumoniae* G10-19 *Stenotrophomonas maltophilia* A04-01, *Pseudomonas aeruginosa* F04-16, and *Lysinibacillus sphaericus* E11_14. This study provided novel information on indigenous hydrocarbon utilizing bacteria associated with *Hibiscus cannabinus* suitable as inoculants for *in situ* bioremediation.

Keywords: *Hibiscus cannabinus*; Hydrocarbon utilizing bacteria; Rhizosphere; Core.

1. Introduction

The Niger Delta Region of Nigeria covers majorly

the South-South Geopolitical Zone of Nigeria [1]. This region has been exposed to hydrocarbon contamination as a result of activities such as oil theft, corrosion and vandalization of pipelines leaving the environment endangered. The presence of hydrocarbons in any environment causes the recruitment of hydrocarbon-utilizing microbes which utilize these hydrocarbons as a sole energy and carbon source. Phytoremediation which is the use of plants and their associated microorganisms is an attractive technology in the removal of contaminants [2]. These plant associated bacteria colonize the rhizosphere (rhizobacteria), phyllosphere (epiphytes) and inside of plant tissues, i.e., endophytes some of which are able to degrade hydrocarbons [3]. The appropriate plant associated bacteria for phytoremediation are those which are tolerant to hydrocarbons and are capable of metabolizing hydrocarbons as their carbon sources for their own growth by using specific hydrocarbon catabolic genes [4]. Similarly, not all plant species are suitable for phytoremediation of petroleum hydrocarbons. A phytoremediator should be tolerant to hydrocarbons, possess an extensive root system and release root exudates that support the degradation of hydrocarbons [5].

Kenaf (*Hibiscus cannabinus* L.) is an established phytoremediator of hydrocarbons. It meets the properties of a suitable plant for phytoremediation by possessing a deep tap root and extensive lateral roots, releases exudates which are alcoholic in nature such as alkaloids, steroids, polyphenolics and saponins [6,7]. The plant favors the establishment of specific bacteria in and around them, these associated bacteria make their way through root cracks, wounds and sites where lateral roots emerge [8,9]. The core of Kenaf (*Hibiscus cannabinus*) is a good absorbent of petroleum, one of its unique features being that it leaches only about 0.02% of it. As it removes these pollutants, they are now made available to the indigenous microbes within it [10]. Agamuthu et al. [11] observed over 83% loss of lubricating oil in the soil after phytoremediation with Kenaf (*Hibiscus cannabinus*) but no accumulation of lubricating oil in the plant's parts, this was attributed probably to the indigenous microbes present which utilize the lubricating oil as a source of energy.

Similarly Adedosun et al. [12] in a bioremediation study using Kenaf (*Hibiscus cannabinus*) suggested its absorption of total petroleum hydrocarbons to be due to the presence of microorganisms which utilized them. In all of these indigenous studies while suggesting the presence of indigenous microbes within this plant there seems to be paucity of information on the identities of these microbes. The objective of this study was to isolate indigenous endophytic and rhizospheric bacteria from Kenaf (*Hibiscus cannabinus*) grown in a hydrocarbon unimpacted soil with the aim of identifying the ones with hydrocarbon utilizing ability.

2. Study Area

Pot experiment was carried out in a provided space within Federal Ministry of Agriculture and Rural Development Rumuodomaya, Port Harcourt Rivers State, Latitude 4053'08.9N and Longitude 7000'12.8E for a period of 17 weeks (February-June, 2016). 20 kg of top soil collected from the site was measured into each pot which was pierced with 15 holes for proper drainage. Cuba 108, Tianung 1 and Tianung 2 accessions of *Hibiscus cannabinus* were planted a set of pots were left unplanted to serve as control. *Hibiscus cannabinus* were obtained from Kenaf Research and Improvement Programme, Institute of Agricultural Research and Training, Moor Plantation, Ibadan, Nigeria. Three (3) seeds per pot were sown at a depth of 1.5-2.0 cm and some pots left unsown as control.

3. Methodology

3.1 Bacteriological analysis of rhizosphere soil samples

Two samples (stem and rhizosphere) were aseptically collected from two plants of setups A, B and C at 3 weeks after germination (here they are juvenile lacking fruits, flowers) and at 17 weeks after germination (here the plants were mature having tiny thorns and flowers). Rhizosphere samples were obtained by uprooting two plants (plant 1 and plant 2) for each accession and shaking the soil that adhered to the roots into sterile bottles. Unplanted soil (set up D) was also collected to serve as a control. These collected rhizosphere and non-rhizosphere control samples were taken to the laboratory for processing within 24 h.

Nutrient agar was used to enumerate heterotrophs while vapour phase transfer method as described by Abu et al. [13] was adopted to enumerate rhizospheric hydrocarbon-utilizing bacteria on mineral salt agar as modified by Opokwasili et al. [14].

3.2 Core processing and bacteriological analysis

Stem samples were collected aseptically into sterile plastic bottles and transported to the laboratory for processing within 4 h. Processing of core was done according to the method of Cao et al. [15]:

To isolate endophytic bacteria the treated stem samples were aseptically cut into bits of 2-3 cm, divided longitudinally and their bark removed using a sterile scalpel. For enumeration of heterotrophic bacteria, the core and bark (serving as plant control) were aseptically plated on nutrient agar plates in duplicates and incubated at 35°C for 24-48 h. Sub culturing was done until pure colonies were obtained. Emulsification test as done by Kostka et al. [16] was used to isolate hydrocarbon utilizing endophytes from the heterotrophic endophytes.

3.3 Cultural, morphological and biochemical characterization of hydrocarbon-utilizing bacterial isolates

Pure cultures of hydrocarbon utilizing bacteria from both the core, rhizosphere and non-rhizosphere soil samples were identified based on their biochemical characteristics, colonial morphology and cell-micromorphology. Indole, Methyl red, Voges-Proskauer, Citrate Utilization, Catalase, Oxidase, Sugar fermentation, Gram stain, Urease, Hydrogen Sulphide production tests were employed for these characterization processes (pp: 83-89). Identities of the hydrocarbon-utilizing bacterial isolates were confirmed using Bergey's Manual of Determinative Bacteriology [17].

3.4 Molecular characterization of hydrocarbon-utilizing bacterial isolates

This involved the following procedures: DNA extraction, 16S rRNA gene amplification, sequencing and identification of obtained DNA sequences according to Chikere et al. [18].

4. Statistical Analysis of Data

One way analysis of variance (ANOVA) SPSS 21.0 was used to analyze total culturable heterotrophic and hydrocarbon-utilizing bacterial counts.

5. Results and Discussion

The results of this study reveal that an apparently uncontaminated soil on which plants grow could house Hydrocarbon-Utilizing Bacteria (HUB). This could be singly or jointly as a result of previous exposure of the soil to accidental spills and the presence of alcoholic components such as polyphenolics, steroids which were excreted as exudates from Kenaf (*Hibiscus cannabinus*) plant [7]. Thirteen (13) hydrocarbon-utilizing bacterial species were isolated from the bark, soil control, core and rhizosphere of Kenaf (*Hibiscus cannabinus*) and belong to phyla of dominant plant associated bacteria as reported by Ryan et al. [19]. *Lysinibacillus* spp. and *Exiguobacterium aurantiacum* belong to the phylum Firmicutes while other species belong to the phylum Proteobacteria.

For both plants 1 and 2, Cuba 108 had the highest mean total culturable heterotrophic bacterial count (TCHBC) and hydrocarbon utilizing bacterial count. The soil control yielded the lowest counts generally because it

did not bear any plant which would have contributed in improved aeration and fertility of soil thus encouraging bacterial proliferation. Also hydrocarbon-utilizing bacterial counts and heterotrophic bacterial counts were higher at maturity than at juvenile stage (Figures 1-5 and Table 1). Nichols et al. [20] after a study suggested that plant's presence stimulates increase in microbial numbers; this was after an observation that the rhizospheric microbial counts of Alfalfa (*Medicago sativa*) and Alpine blue grass (*Poa alpma*) were higher than the non rhizospheric microbial counts of unplanted soil. Gonzalez-Franco et al. [21], in a study with grass samples taken from January to April in California observed higher microbial counts in older grasses of big sage brush (*Artemisia tridentate*) than in younger ones, rhizospheric soil was also observed to have more bacterial counts than bulk soil. At maturity metabolic activity is increased processes like photosynthesis take place Carbon and organic acids fixed by plant photosynthesis are translocated into the root zone via rhizodeposition [22,23]. They are later released as exudates from the root hairs and act as chemical signals for bacteria to move by chemo taxis to the surfaces of roots. Hydrocarbon-utilizing bacterial

counts were lower than heterotrophic bacterial counts. Hydrocarbon-Utilizing bacteria depend on oil as the only source of carbon and energy while heterotrophs utilize every available source of carbon encouraging the growth of a wide range of heterotrophic bacteria. Other observations in this study were the dominance of a particular bacteria genus at the various stages of sampling (*Providencia* spp. at juvenile stage and *Lysinibacillus* at maturity), the presence of similar species in the rhizosphere and control samples (bulk soil), the presence of similar in the Littler and rhizosphere samples and the presence of bacteria in the bark of *Hibiscus cannabinus* plant. For all of these observations there are possible explanations. The presence of similar species in the rhizosphere and bulk soil (control) could mean that these bacteria were derived from the bulk soil but migrated (with the aid of flagella and fimbriae) as a result of the chemical attractants in the root zone. The presence of similar microbes the core and rhizosphere (*Providencia rustigianii* H10_22 and *Pseudomonas aeruginosa* F04_16) may corroborate the opinion by Compant et al [24] that endophytic microbes are majorly a subset of the rhizosphere population. Factors like competition

Table 1. Physical and molecular characteristics of HUB isolates.

Isolate code	HUB strain	Source	Stage of Sampling	NCBI Data Base Match
AT1	<i>Providencia vermicola</i> A10_01	c	Juvenile	<i>Providencia vermicola</i>
AT2	<i>Providencia rettgeri</i> B10_04	r and sc	Juvenile	<i>Providencia rettgeri</i> BPES6
AT3	<i>Providencia rettgeri</i> D04_10	sc and r	Juvenile	<i>Providencia rettgeri</i> 11TRP2
AT4	<i>Pseudomonas aeruginosa</i> D10_10	b	Juvenile and Maturity	<i>Pseudomonas aeruginosa</i> CIFRIDTSBI
AT5	<i>Exiguobacterium aurantiacum</i> F03_18	b	Juvenile and Maturity	<i>Exiguobacterium aurantiacum</i> SPD2
AT6	<i>Providencia vermicola</i> F10_16	c	Juvenile	<i>Providencia vermicola</i>
AT7	<i>Klebsiella pneumoniae</i> G10_19	r	Juvenile	<i>Klebsiella pneumoniae</i> 77_2
AT8	<i>Stenotrophomonas maltophilia</i> A04_01	r	Juvenile	<i>Stenotrophomonas maltophilia</i> JAK_10
AT9	<i>Providencia rustigianii</i> H10_22	r and c	Maturity	<i>Providencia rustigianii</i> NCTC11802
AT10	<i>Lysinibacillus fusiformis</i> B11_05	sc and r	Maturity	<i>Lysinibacillus fusiformis</i> ANA83
AT11	<i>Lysinibacillus sphaericus</i> C11_08	r	Maturity	<i>Lysinibacillus sphaericus</i>
AT12	<i>Pseudomonas aeruginosa</i> F04_16	c and r	Maturity	<i>Pseudomonas aeruginosa</i> CI-05
AT13	<i>Lysinibacillus sphaericus</i> E11_14	r and sc	Maturity	<i>Lysinibacillus sphaericus</i>

C: Core; r: Rhizosphere; sc: Soil Control; b: Bast

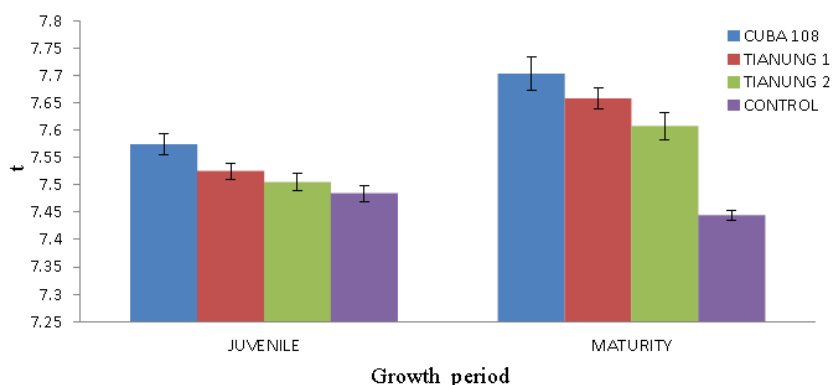


Figure 1. Population density of HUB obtained from the rhizosphere of sample plant 1 between juvenile and maturity periods.

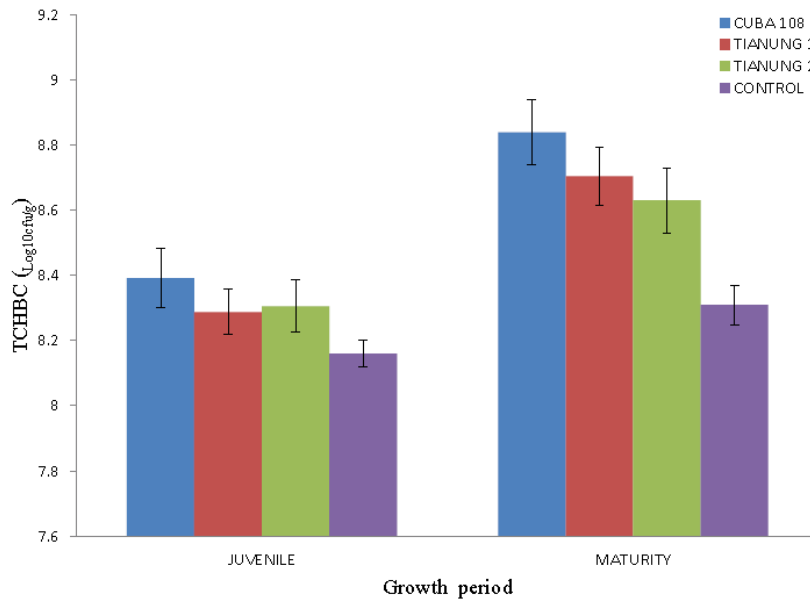


Figure 2. Population density of TCHBC obtained from the rhizosphere of sample plant 1 between juvenile and maturity periods.

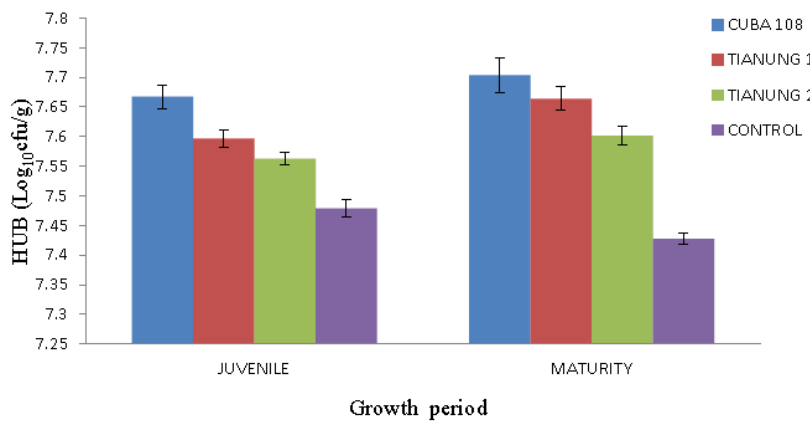


Figure 3. Population density of HUB obtained from the rhizosphere of sample plant 2 between juvenile and maturity periods.

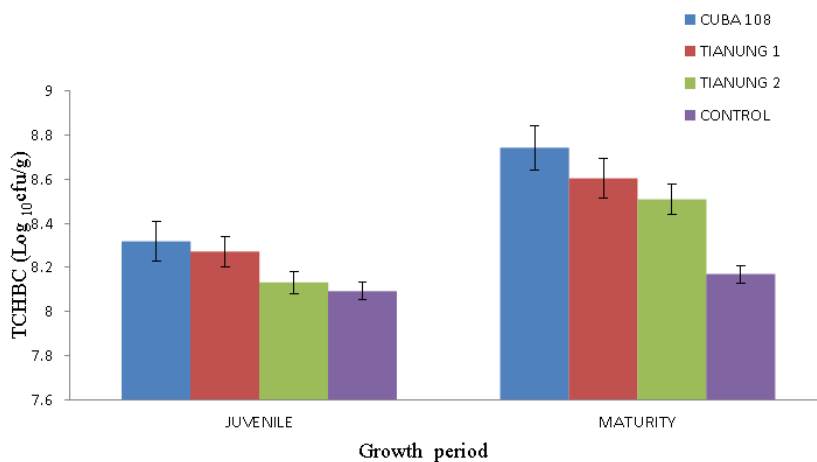


Figure 4. Population density of TCHBC obtained from the rhizosphere of sample plant 2 between juvenile and maturity periods.

for space, nutrients may have brought about migration of some species to the core (Figure 1).

HUB found in the bark of *Hibiscus cannabinus* plant

could have been from wounds caused by insects and nematodes, recall nematodes are a major pathogen of *Hibiscus cannabinus* plant. HUB were more in the rhizosphere as nutrients used by plants

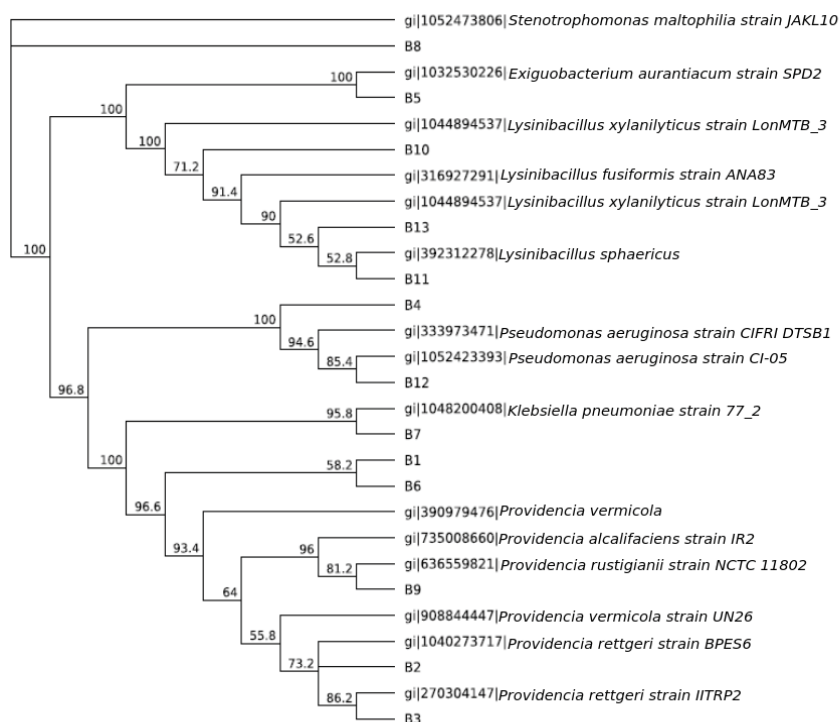


Figure 5. Neighbour joining tree showing evolutionary relationship between HUB isolates and close relatives.

are usually conducted from the root to the shoot making the root system the warehouse for food and water. *Lysinibacillus* spp. is a spore bearing bacteria and spore formation, is an adaptation to harsh environmental conditions, this may explain its absence at the juvenile stage of growth. *Providencia vermicola* has been isolated from infective juveniles of the entomopathogenic nematodes [25]. It is therefore possible that *Providencia* spp. are borne by these nematodes which are pathogenic to *Hibiscus cannabinus* plant. *Lysinibacillus fusiformis*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* have been reported to produce volatile organic components such as ketones and amines which are nematocidal this may have suppressed the *Providencia* spp. population especially at maturity [26,27].

Apart from *Providencia rustigianii* for which there is no previous documented isolation of it from plant environment, the other isolated hydrocarbon utilizing bacteria in this study, are documented rhizosphere and endophytic associated bacteria and hydrocarbon-utilizing bacteria [28-36]. Little is known about the functional diversity of plant associated microbes [37-53].

6. Conclusion

Based on the research carried out it can be suggested that hydrocarbon-utilizing bacteria exist in crude oil unimpacted soil Cuba 108 accession of *Hibiscus cannabinus* may be an excellent reservoir for bacteria associated with *Hibiscus cannabinus* plant since it provided higher counts for both hydrocarbon-utilizing bacteria and heterotrophic bacteria in this

study. *Providencia* spp. is probably a dominant hydrocarbon-utilizing bacterial species associated with *Hibiscus cannabinus* due to its presence at both stages of sampling and in all samples tested.

Cultures of these isolated hydrocarbon-utilizing bacteria could be grown in the laboratory for use in bioremediation of crude oil-impacted media. The cultivation of *Hibiscus cannabinus* in the Niger Delta would lead to the production of natural sorbents for use in oil spill cleanup and the indigenous bacteria would biodegrade the crude oil leading to the bioremediation of the crude oil impacted media.

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