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Morphological and Molecular Characterization of Endophytic Bacteria Isolated from Bambara Groundnut (*Vigna subterranea*) Nodules in Daloa, Côte d'Ivoire

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

A thorough understanding of the diversity, functions and specific interactions of endophytic bacteria present in legumes is essential for improving crop production. This study aimed to identify roots nodule endophytes associated to Bambara groundnut by analyzing their morphological diversity and genetic diversity. A total of 34 bacterial isolates were sampled from different cultivation sites and associated with 5 local varieties of Bambara groundnut.

Morphological analysis using macroscopic and microscopic observation revealed that the bacterial isolates were morphologically diverse in terms of colony appearance, shape, colony size, color, opacity and Gram stain result. These bacteria exhibited characters different from those of the symbiotic bacteria. In addition, molecular identification based on sequencing the 16S-rRNA gene and 1081 bp analysis showed the existence of non-symbiotic bacteria in Bambara groundnut nodule. The community of roots nodule endophyte isolated from Bambara groundnut belonged to the genera *Pseudomonas, Bacillus, Bacterium, microbacterium, Rahnella, Paenibacillus, Lysobacter* with 98 to 99.5% similarity, classified under the *Firmicutes, Actinobacteria* and *Proteobacteria* phyla. In Bambara groundnut, the predominant nodule endophytes were *Bacillus* (56%) and *Pseudomonas* (17%).

Future research could further investigate the ecological implications of these bacterial interactions, and their potential applications in biotechnology and agronomy, particularly in biofertilization and host plant growth promotion.

Keywords: Endophyte; Genetic diversity; morphological diversity; Bambara groundnut.

1. INTRODUCTION

Bambara groundnut (*Vigna subterranea* L.) is an indigenous African food legume highly tolerant to drought, salinity and infertile soil with enormous potential in nutrition and soil fertilization (Taffouo et al., 2010). Few researches carried out have reported that *Vigna subterranea* L. Verdc is nodulated by *Bradyrhizobium* (Ibny et al., 2019) and *Rhizobium pusense* (Gnangui et al., 2019). In addition to nodulating bacteria, legumes nodules shelter other non-nodulating bacteria called endophytes (Peix et al., 2015). *While the legume–Rhizobium interaction is highly specific, research has shown that non-rhizobial rhizobacteria can also colonize the selective nodule environment*. Roots nodules which traditionally were considered as the exclusive niche of rhizobia are being revisited to examine colonization by several non-symbiotic bacteria unrelated to symbiotic nitrogen fixation. Evidence suggest that the healthy nodule can contain endophytes not necessarily related to symbiotic or diazotrophic context has been documented, Examples include *Bacillus* in soybean (Bai et al., 2002), Klebsiella in groundnut, clover, and bean (Ozawa et al., 2003), and Pseudomonas in acacia (Kuklinsky-Sobral et al., 2004) and soybean (Hoque et al., 2011). Sturz et al. (1997) simultaneously recovered 4.3×10^9 CFU rhizobia and 3×10^5 CFU non-rhizobial endophytes of 12 bacterial genera per gram fresh weight of red clover nodule tissue. Evidence that healthy nodule interiors of wild legumes can contain bacteria not related to rhizobia has also been reported (Zakhia et al., 2006; Deng et al., 2011). Endophytic bacteria, which reside within the nodules of legume plants, play a vital role in improving plant nutrition, thus giving them a distinct advantage in terms of growth and development (Gasser, 2022). In addition, the presence of these endophytic bacteria promotes resistance to abiotic and biotic stresses, thus providing valuable protection to host plants. (Maamri, 2023). It is essential to have a thorough understanding of the diversity, functions, and specific interactions of endophytic bacteria present in legumes particularly, Bambara groundnut in order to improve production of this crop. Furthermore, such knowledge would allow the development of innovative biotechnology strategies with the aim of promoting sustainable and environmentally friendly agriculture. Therefore, the aim of this study was to identify the endophytic bacteria of the Bambara groundnut nodule by analyzing their morphological diversity and genetic diversity.

2. MATERIALS AND METHODS

The bacteria were isolated *in vitro* at the Jean Lorougnon Guédé University in Daloa, Côte d'Ivoire. These isolates were tested for their genetic diversity in a laboratory at Mohammed V's Faculty of Science in Rabat, Morocco.

2.1 Sampling Site

Five sites were investigated for soil sampling in the localities of Daloa in Côte d'Ivoire, including Bribouo, Toroguhé, Jean Lorougnon Guedé University, Zakoua, and Zépréguhé (Guei et al., 2020).

2.2 Isolation of Bacterial Strain from Nodules

The bacteria were isolated from soil samples collected from five varieties of Bambara groundnut. seeds. Plants were grown from sterile seeds in plastic pots with soil, which were regularly watered. Nodules were harvested from these plants.

Roots nodules were surface sterilized by washing for 3 min with 70% ethanol, immersed in 30% sodium hypochlorite for 2 min and finally were washed six times by sterile water. 1 mL of each ground nodule was spread on the solid YEM medium (Vincent, 1970) supplemented with 0.025 g/L of Red Congo. The cultures were incubated at 28°C for 3-6 days (Somasegaran & Hoben, 1994**)**. After incubation, the colonies obtained were purified by streaking technique according to the Jordan method and stored in 20% (v /v) of glycerol at -80°C (Guei et al., 2020).

2.3 Morphological Characterization of Bacterial Isolates

The purified colonies were subjected to macroscopic observation according to the Jordan (1984) method which consisted of analyzing the s shape, size, chromogen, opacity, elevation, surface and consistency of the colonies. Also, these bacteria were examined microscopically to determine their Gram type, their shape and their grouping mode according to the method used by Filloux & Vallet (2003).

2.4 Molecular Characterization of Bacterial Isolates

2.4.1 Bacterial DNA isolation

The total DNA of bacterial isolates was extracted using phenol-chloroform and RNase treatment from pure cultures during the phase of exponential growth in YEM medium. The isolation of pure DNA has carried out according to the Chen & Tsong-teh (1993) method in a volume of 300 µL of bacterial lysis buffer: (40 mM Tris acetate (pH 7.8), 1 mM EDTA, 1% SDS, 20 mM sodium acetate and RNase at 20 mg/ml) and 100 µL of 5M NaCl. The pellet resulting from centrifugation was washed with 100% and 70% ethanol after purification with the Phenol-Chloroform mixture (v/v). Then, it was suspended in 55 µL of TE (pH 7.8, 10 mM Tris, 1 mM EDTA) and kept at -20°C. The NanodropTM Spectophotometer measured the quantity and quality of DNA using 260 nm for DNA and 280nm for protein. The PCR reactions were performed with 20 ng of DNA from each bacterial isolate (Guei et al., 2020).

2.4.2 PCR amplification of the 16S-rRNA gene and sequencing

To amplify the 16S rRNA gene, a universal primer pair, fD1 (5´-AGAGTTTGATCCTGGCTCAG-3´) and rD1 (5´AAGGAGGTG ATC CAG CC-3´) (Weisburg et al., 1991) was used. PCR amplification reactions were performed according to the method described by Guei et al. (2020). Partial 16S rDNA sequencing was performed from the amplification products. These amplifies were purified with the Qiagen PCR Product Purification Kit and then sequenced using the same primers as for PCR. A 3130xl automated sequencer was used to analyze the products at the National Center for Scientific and Technical Research (CNRST) in Rabat, Morocco.

2.4.3 Sequences alignment and phylogenetic analyses

To conduct phylogenetic analyses on bacteria isolated from Bambara groundnut nodules based on the sequences obtained, the verification of the quality of the sequences, alignment of the sequences, and construction of the phylogenetic tree were executed according to the methodology employed by Guei et al. (2020).

3. RESULTS

3.1 Morphological Characters of Bacteria Isolated from Bambara Groundnut Nodule

Sixty (60) microorganisms were isolated from Bambara groundnut root nodules grown in five distinct soil types. No bacterial growth was observed on the YEM medium inoculated with the water used from the final rinse of the nodule. The bacterial isolates were phenotypically diverse in terms of colony appearance, shape, size (diameter), color and opacity (Fig. 1). In the following, 34 bacteria presenting characters different from those of the symbiotic bacteria will be considered.

Macroscopic observation of colonies on Petri dishes revealed that most isolates were spherical or round in shape. They are translucent, transparent or opaque with a regular, filamentous or crenate outline and of variable color. Colonies had a smooth appearance, with a predominant flat or domed elevation and a diameter ranging from 1 to 4 mm (Table 1).

Microscopic analysis revealed that the majority of the bacteria isolated were Gram-positive, with a small number of negative-positive bacteria, bacilli, and cocci with a variety of grouping modes (Fig. 2, Table 1).

3.2 Phylogenetic Analysis of the 16S-rRNA Gene

The genomic region of 16S rRNA of these 34 bacteria was successfully PCR amplified using the fD1 and rD1 primers. The sequencing of these 16S rRNA gene fragments (ca. 1500 bp) and the computer analysis of their data revealed that these isolates are root nodule endophytes (non-symbiotic bacteria).

The partial sequences of the 16S rRNA (1058–1116 bp) remove it as the data in Table 1 does not match here, were obtained and deposited in the GenBank® database under the accession numbers MT661489 to MT661522 (Table 2). According to the 16S rRNA genetic similarity, bacterial isolates were closely related to seven (07) genera (*Pseudomonas, Baccillus, Microbacterium, Rahnella, Paenibacillus, Lysobacter, Curtobacterium)* distributed in three (03) phyla (*Firmicutes, Actinobacteria and Proteobacteria)*. In Groundnut Bambara, the predominant nodule endophytes were *Baccillus* (56%) and *pseudomonas* (17%) (Table 1).

Fig. 1. Morphological characteristics of bacterial isolates from *Bambara groundnut* root nodules

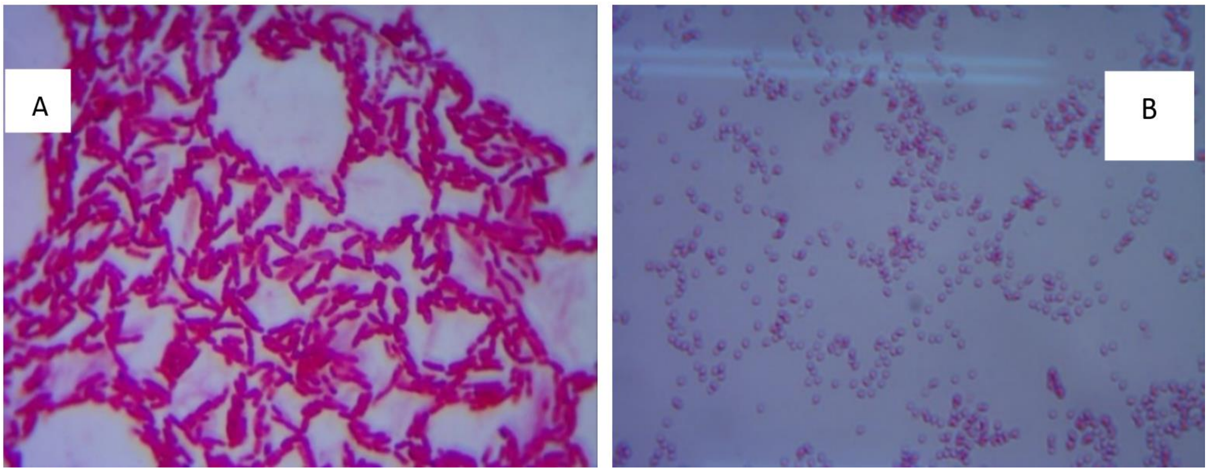

Fig. 2. Microscopic observation at 100X magnification after Gram staining (A: Gram-positive bacteria, bacilli; B: Gram-positive bacteria, cocci)

Table 1. Morphological characters of bacteria isolated from *Vigna subterranea*

	Morphological characteristics of colonies							Morphological characteristics of isolates		
Isolates	**Shape**	**Chromogen**	**Surrounding**	**Elevation**	**Size (mm)**	**Opacity**	**Appearance**	**Gram**	**Shape**	**Gouping**
RFK1	Spherical	Cream	Régular	Cambered	2 to 3	Transparent	Smooth	+	bacilli	secluded
RFK2	Spherical	Orange	Régular	Flat	1 to 4	Translucent	Smooth	+	bacilli	secluded
RFK3	Spherical	Orange	serrated	Flat	≤1	Translucent	Rough	+	bacilli	chain
RFK4	Round	Pink	Régular	Plat	<2	Opaque	Smooth	+	bacilli	secluded
RFK5	Spherical	Cream	Régular	Cambered	≤4	Opaque	Smooth	+	bacilli	diplo
RFK6	Cirular	Cream	Régular	Cambered	3 to 4	Opaque	Smooth	+	bacilli	Tétrad
RFK7	Cirular	Cream	Régular	Convex	1 to 4	Translucent	Smooth	+	bacilli	secluded
RFK8	Point	Cream	Régular	Raised	≤1	Translucent	Rough	+	bacilli	Heaps
RFK9	Cirular	Orange	Régular	Cambered	3 à 4	Translucent	Smooth	+	bacilli	secluded
RFK10	Cirular	Orange	Régular	Flat	<3	Translucent	Smooth	+	bacilli	Heaps
RFK11	Cirular	Orange	Régular	Raised	1 to 5	Translucent	Smooth	+	bacilli	secluded
RFK12	Point	Orange	serrated	Flat	<2	Translucent	Smooth	+	bacilli	chain
RFK13	Spherical	Orange	Régular	Flat	2 to 4	Opaque	Smooth	+	bacilli	Heaps
RFK14	Round	Cream	Régular	Flat	< 2	Transparent	Smooth	+	bacilli	secluded
RFK15	Cirular	Cream	Régular	Raised	1 to 4	Opaque	Smooth	+	bacilli	Heaps
RFK16	Round	Pink	Régular	Flat	≤2	Opaque	Smooth	+	cocci	secluded
RFK17	Round	Pinkish	Régular	Flat	<1	Transparent	Smooth	+	bacilli	secluded
RFK18	Spherical	Cream	Régular	Raised	1 to 2	Translucent	Smooth	+	bacilli	Heaps
RFK19	Spherical	Cream	Régular	Flat	2 to 3	Translucent	Smooth	+	bacilli	diplo
RFK20	Spherical	Cream	Régular	Raised	1 to 2	Translucent	Smooth	-	bacilli	Heaps
RFK21	Spherical	Pink	Régular	Flat	1 to 2	Opaque	Smooth	-	bacilli	secluded
RFK22	Cirular	Pinkish	Régular	Raised	2 to	Opaque	Smooth			secluded
RFK23	Round	Pink	Régular	Flat	≤ 2	Translucent	Smooth	-	bacilli	secluded
RFK24	Round	Cream	Régular	Flat	≤ 2	Opaque	Smooth	-	bacilli	Heaps
RFK25	Sphérique	Orange	Régular	Convex	1 to 3	Translucent	Smooth	-	bacilli	diplo
RFK26	Cirular	Orange	Régular	Raised	1 to 3	Opaque	Smooth	-	bacilli	secluded
RFK27	Spherical	Orange	Régular	Flat	2 to 4	Opaque	Smooth	-	bacilli	Heaps
RFK28	Round	Orange	Régular	Flat	≤2	Translucent	Smooth	-	bacilli	chain
RFK29	Round	Yellow	Régular	Flat	≤ 2	Translucent	Smooth	-	bacilli	diplo
RFK30	Spherical	Cream	Régular	Flat	2 to 3	Transparent	Smooth	-	bacilli	diplo
RFK31	Spherical	Cream	Régular	Flat	2 to 3	Transparent	Smooth	+	bacilli	diplo
RFK32	Spherical	Cream	Régular	Flat	2 to 4	Opaque	Smooth	+	bacilli	diplo
RFK33	Spherical	Cream	Régular	Flat	<4	Transparent	Smooth	-	bacilli	diplo
RFK34	Cirular	Pinkish	Régular	Flat	2 to 4	Translucent	Smooth	+	bacilli	Chain

Table 2. Séquences analysis of 16S rRNA from nodule endophytes

Strains	Sequences (bp)	Accession number	Homology to the reference strains		Similary(%)
RFK1	1082	MT661489	*Bacillus megaterium* strain FBMAX18	MK791705	98,04
RFK2	1086	MT661490	Bacillus sp. L105	DQ248043	99,42
RFK3	1116	MT661491	Bacillus sp. L105	DQ248043	98,89
RFK4	1107	MT661492	*Bacillus safensis* strain BXC22	MN227495	98,09
RFK5	1080	MT661493	*Bacillus nealsonii* strain ASB-160	MK514991	98,18
RFK6	1083	MT661494	*Bacillus megaterium* strain YN7 16S	MK961265	98,77
RFK7	1094	MT661495	Bacillus sp. BAB-3563	KJ938561	98,86
RFK8	1080	MT661496	*Bacillus subtilis* strain THt3-1	HQ333014	98,38
RFK9	1086	MT661497	*Bacillus cereus* strain THt1-8	HQ333012	98,6
RFK10	1103	MT661498	*Bacillus subtilis* strain V90	HQ268534	98,6
RFK11	1089	MT661499	*Bacillus pocheonensis* strain P12	JN700154	98,86
RFK12	1084	MT661500	*Bacillus cereus* strain BCRh6	KT153602	98,38
RFK13	1089	MT661501	*Bacillus cereus* strain BAB-6399	MF351779	98,27
RFK14	1089	MT661502	*Bacillus subtilis* subsp. subtilis strain GC17	KC955127	99,17
RFK15	1085	MT661503	*Bacillus tequilensis* strain RBB6	MN032396	99,55
RFK16	1092	MT661504	*Bacillus megaterium* strain VITNJ1	MH100903	98,63
RFK17	1081	MT661505	*Bacillus megaterium* strain E2-04	MN525604	98,61
RFK18	1078	MT661506	*Bacillus megaterium* strain DS8	EU835733	98,87
RFK19	1086	MT661507	*Bacillus subtilis* strain LWIS15	KT945024	98,8
RFK20	1099	MT661508	*Bacillus subtilis* subsp. subtilis strain AN3	MN560000	98,75
RFK21	1093	MT661509	Pseudomonas sp. strain Fas20	MH235977	98,98
RFK22	1100	MT661510	Pseudomonas sp strain 7	MK322941	98,85
RFK23	1113	MT661511	*Pseudomonas azotoformans* strain R2SsM3P2C7	KF147042	98,86
RFK24	1099	MT661512	Pseudomonas sp. strain Fas20	MH235977	98,9
RFK25	1082	MT661513	*Pseudomonas koreensis* strain PS3TA2	KY910138	98,33
RFK26	1104	MT661514	*Pseudomonas fluorescens* strain FC6846	MH497588	98,73
RFK27	1100	MT661515	*Pseudomonas poae* strain 15-A1	MN307356	98,87
RFK28	1083	MT661516	Lysobacter sp. KNUC361	EU239150	99,5
RFK29	1070	MT661517	Lysobacter sp. KNUC361	EU239150	98,77
RFK30	1115	MT661518	*Rahnella inusitata* strain FOD 9/21	KF308406	98,61
RFK31	1089	MT661519	Microbacterium sp. HBUM179633	KR906409	98,34
RFK32	1101	MT661520	Microbacterium sp. strain JL3592	KX989137	98,61
RFK33	1058	MT661521	*Paenibacillus lautus* strain E118	JF683659	98,93
RFK34	1081	MT661522	*Curtobacterium pusillum*	LN681569	99,25

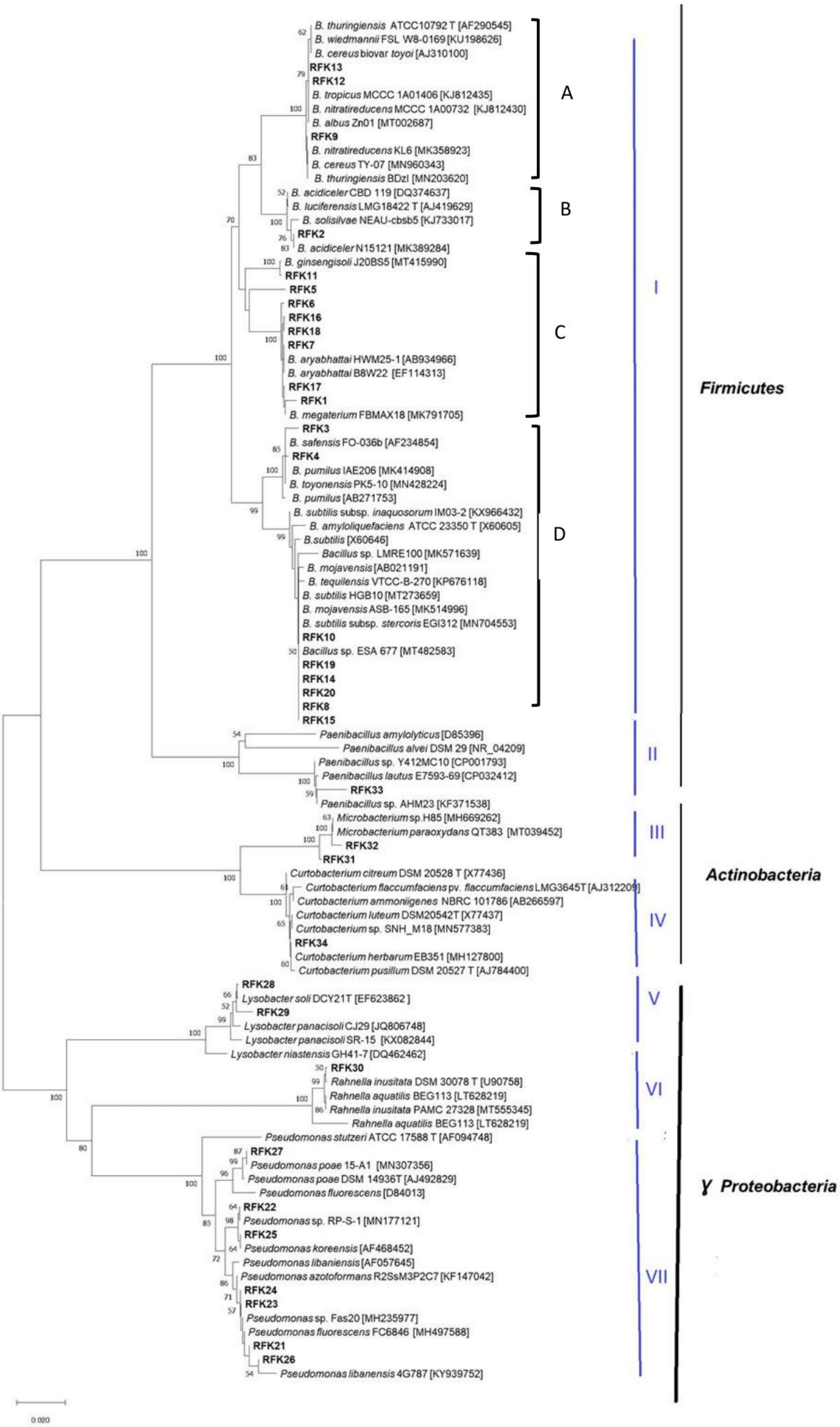

Fig. 3. Phylogenetic tree based upon the 16S rDNA sequences obtained showing the phylogenetic positions of endophytes isolated from Bambara groundnut root nodules

The phylogenetic tree representing different species of endophyte reference strains including local isolates was built by the neighborhood (NJ) (Fig. 3). The representative isolates clustered together with the corresponding most related type strains These local isolates were distinctly divided into seven clusters.

Cluster I, which contains the *Bacillus* species, is divided into four distinct subgroups.

Subgroup A includes that strains RFK 9, RFK12, and RFK13 are related to nine species of the *Bacillus* genus.

Subgroup B revealed that strain RFK2 is very close to the species *B. acidiceler* N15121.

Subgroup C contained four references strains of Bacillus species and showed 100% similarity between strain RFK11 and species *B. ginsengisoli*, between strains RFK15, RFK6, RFK16, RFK18, RFK7 and species *B. aryabhattai* and then between strains RFK 17, RFK1 and species *B. megaterium* FBMAX18.

Subgroup D demonstrated a strong genetic link between strain RFK3 and the species *B. toyonensis*, strain RFK4 and the species *B. pumilus,* and then strains RFK10, RFK19, RFK14, RFK20, RFK8, RFK15 and six species of the genus *Bacillus*.

Cluster (II) revealed that strain RFK33 is remarkably similar to the species *Paenibacillus lautus* E7593-69.

Cluster (III) includes strains RFK31 and RFKC32, which ’are closely related to the species *Microbacterium paraoxydans* QT383.

Cluster (IV) includes strain RFK34 which is genetically related to the species *Curtobacterium* sp. SNH_M18.

Cluster (V) found that strains RFK28 and RFK29 are very close to the species *Lysobacter soli* DCY21T.

Cluster (VI) includes strain RFK30, which bears a resemblance to the species *Rahnella inusitata* DSM 30078 T.

Finally, the seventh (VII) cluster revealed a very close genetic link between strain RFK27 and the species *Pseudomonas poae* 15-A1, and between strain RFK22 and *Pseudomonas* sp. Between strain RFK25 and the species *Pseudomonas koreensis*, strains (RFK 23 anadRFK24) and the species *Pseudomonas azotoformans*, RP-S-1 between strain RFK21 and the species *Pseudomonas fluorescens*, between strain RFK26 and the species *Pseudomonas libanensis* 4G787.

Cluster I (*Bacillus*) and Cluster II (*Paenibacillus*) included in the *Firmicutes* phylum, Cluster III (*Microbacterium*) and Cluster IV(*Curtobacterim*) included in the *Actinobacteria* phylum and Cluster V (*Lysobacter*)*,* Cluster VI (*Rahnella*) and Cluster VII (*pseudomonas*) included in the *Proteobacteria* phylum.

4. DISCUSSION

A total of 60 nodule bacteria isolates were obtained from the five soil samples collected from five Daloa localities in Côte d’Ivoire. All of the nodules that were sampled were healthy, as evidenced by their pink hue and firm texture. Therefore, the endophytic bacteria were not exploiting the decay of nodules. The absence of bacteria in the water used in the final rinse of the nodule surface was evidence that their soil contaminants were not present (Guei et al., 2020).

The 34 bacterial strains in our collection exhibit morphological and molecular diversity, and their distinguishing characteristics set them apart from symbiotic bacteria. This fact indicates that the Bambara groundnut nodules harbor endophytic bacteria, which are non-symbiotic. this observation could be accounted for by the endophytes presence in the soils where the plants were grown. Indeed, the environment is the primary source of endophyte acquisition (Hardoim et al., 2012). Similar observations have been made in studies on soybean (Li et al., 2008) and *Vigna* species (Pandya et al., 2013; Chidebe et al., 2018). Also, Ibny et al. (2019). reported that out of the 201 isolates from nodule Bambara groundnut subjected to nodulation assay, 106 isolates (53% of isolates) failed to nodulate the host plant. The community of root nodule endophyte isolated from Bambara groundnut belonged to the genera *Pseudomonas, Bacillus, Curtobacterium, Microbacterium, Rahnella, Paenibacillus* and *Lysobacter*. It confirms the results reported by several authors which said that different genera of endophyte were associated with soybean (Kuklinsky-Sobral et al., 2004), *Vigna subterranea* (Ibny et al., 2019), *Vigna radiata*

(Pandya et al., 2013) and *Vigna unguiculata* (Chidebe et al., 2018). Previous studies have revealed a high diversity of endophytic bacteria present in legume nodules, such as pea, alfalfa and soybean (Daoudi et al., 2022). The predominant nodule endophytes in Bambara groundnut were *Bacillus*, followed by *Pseudomonas.* These results are consistent with those reported by Ferchichi et al. (2019) who found that *Pseudomonas* and *bacillus* genera were dominant in the nodule of *L. luteus* and *L. angustifolius*. Compan et al. (2010) reported that the *Bacillus* group with strong environmental adaptability is a typical colonizer of various crops. The predominance of *Pseudomonas* in the nodule suggest rhizosphere population-based selection, as it is profusely present in rhizosphere and establishes easily in a wide variety of niches (Spiers et al., 2000). Similarly, Brígido et al. (2019) demonstrated that *Enterobacter* and *Pseudomonas* are the dominant genera in chickpea plants, exhibiting noteworthy capacities for the production of ammonia and indole acetic acid, which are crucial traits for promoting plant growth. Other researchers highlighted the capacity of endophytes associated with Bambara pea to reduce water stress through the PGP activities carried out (Oviya et al., 2023) It was also noted that endophyte management has the potential to enhance legume production, thereby highlighting the significance of a comprehensive approach in crop management (McElveen, 2019).

The diversity of endophytic bacteria and their distribution in different environments could open the way to applications in agriculture and biotechnology for crop improvement and sustainable management of agrosystems.

5. CONCLUSION

The nodules of the Bambara groundnut contain non-symbiotic bacteria called endophytes. These bacteria exhibited both morphological and genetic diversity, as evidenced by their affiliation with seven (07) genera and three (03) phyla. Notably, the genera *Bacillus* and *Pseudomonas* are predominant in Bambara groundnut nodules.

It would be intriguing to carry out additional research in the future to examine the ecological implications of these bacterial interactions as well as possible uses in biotechnology and agronomy, especially with regard to biofertilization and host plant growth promotion.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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