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Promiscuous Symbiotic Interaction between *Rhizobium* and *Glycine max* (Soybean) in Tropical Soils Southwest Nigeria

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Abstract

Tropical soil samples were collected from three different locations Southwest, Nigeria. The first location was Lagos State University: Faculty of Science, Faculty of Management Science and Lagos State University second gate. The second location was Covenant University: soil was collected from the College of Science and Technology, College of Developmental Studies, Lecture theatre and the Covenant University farm. The third location: soil samples were collected from Shagamu. All soil samples were collected at a depth of 0 cm to 30 cm using a hand trowel and were transported to the laboratory in sterile polythene bags. Control soil samples were obtained from Badagry beach in Lagos.

The purpose of this study is to isolate native *Rhizobium sp.* from tropical soils southwest Nigeria with the ability to nodulate the legume *Glycine max* (Soya beans). Serial dilution of the soil samples following aseptic processes generated dilution factors 10^{-4} and 10^{-5} used as inocula for the pour plate method using Nutrient Agar (NA) and Yeast Extract Mannitol Agar (YEMA) as growth media. The plates were incubated at 28 ± 2°C for 48 h which made visible growth to be observed. Visible differences between the numbers of colonies of dilution factors 10^{-4} and 10^{-5} were observed on both NA and YEMA after the colonies were counted. Physical examination of the culture plates showed that all of the colonies appeared round, mucoid and 58.3% of the colonies were creamy white on YEMA. Microscopic examination revealed motile Gram–negative rods on YEMA while 50% of the isolates on NA were non-motile rods. The mean *Rhizobium sp.* population detected in the soil samples was 0.33×10^{-5} CFU/g.

Conclusively, the presence of indigenous rhizobia population specific for Soya bean in the soils evident by nodulation of the legume 8 Week after Planting (WAP) confirms both the symbiotic relationship and its relevance to sustainable agricultural practice.

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Introduction

Rhizobia (singular, "*Rhizobium*") are Gram-negative bacteria that selectively infect legume roots and form root nodule that fix atmospheric nitrogen into ammonia fertilizer for the plant symbiont (legume). Soybean is the world's most important seed legume and its oil makes up about 25% of the global comestible oil and about two-thirds of the world's protein concentrate included in feed for livestock [1]. Legumes possess the ability to form a nitrogen-fixing symbiosis with *Rhizobium sp.* Leguminous plants range from herbaceous species such as cowpea, soybeans, lupin peas, groundnut, peanuts, lentils, and chickpeas to woody species such as *Leucaena leucocephala* [2,3]. *Rhizobia* include *Rhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium* and *Bradyrhizobium* species [4]. Legumes have a very crucial role in the traditional diets of many communities around the world, unlike in Western countries where beans make up a very minor part of their diet, however, its low fat content and the fact that it is a great source of protein, dietary fiber, and an array of micronutrients and phytochemicals makes them essential for healthy living [5,6].

Glycine max (L.) *Merr* commonly known as soybeans is a crop necessary for human and animal consumption because it contains about 40% protein and 20% oil [7]. The United States of America took up cultivation of soybean in 1950s with rapid development and they are currently the world's largest producer of soybean [8]. In the world, it is the crop with the highest protein content and total vegetable oil produce with approximately 40% protein content and 20% oil content [8]. Soybeans are consumed regularly in Asian countries in form of soymilk, tofu and fermented products like sufu, tempeh and miso [9]. It contains a large amount of isoflavones which is a secondary

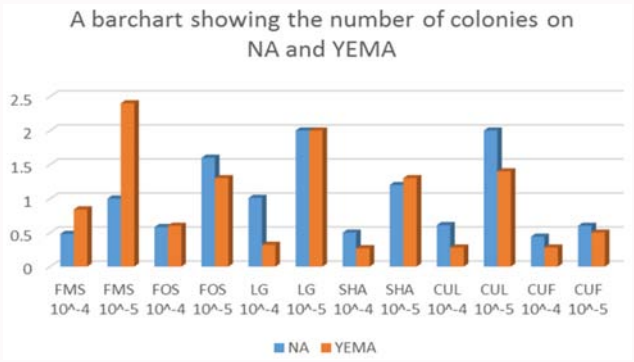

Figure 1: Bar chart showing the number of colonies on NA and YEMA.
The bar chart above shows the pictorial representation of the converted values of the number of colonies derived from the serial dilution of the various samples to CFU/g.
NA: Nutrient Agar; YEMA: Yeast Extract Mannitol Agar; FMS: Faculty of Management Science; FOS: Faculty of Science; LG: LASU (Lagos State University) Gate; SHA: Shagamu; CUL: Covenant University Locations

metabolite [7]. It contains 97% to 98% of flavone glycoside and 2% to 3% of aglycones which gives it the ability to inhibit leukemia, transformation and multiplication of cancerous cells that may affect parts of the body like prostate, lung, breast, and colon [8]. In general, diverse rhizobia belonging to *Bradyrhizobium* and *Sinorhizobium* are associated with Soybeans and typical biogeography patterns have been found among the soybean rhizobia [2,10]. The distribution, abundance and N-fixing efficiency of soybean *rhizobia* are strongly related to genotypes or cultivars of soybeans, latitude and edaphic factors [11,12]. *Bradyrhizobium* species which are the common microsymbiont for soybeans has many genospecies. In cultivated farmlands depending on the different land use and culture histories, presence of cultivated soybeans increased the rhizobial abundance and diversity in cultivated farmlands. It therefore means that different land uses and crop management alone does not alter the diversity and abundance of soybean microsymbiont but change interactions between *rhizobia* and legume or non-legume plant does [13].

Objectives

The objectives of the current study are:

- To determine the population of indigenous *Rhizobium sp.* with the ability to nodulate soybean roots in the various soil samples;

- To determine the morphological characteristics of the *Rhizobium* isolates;

- To differentiate the isolates based on their biochemical properties.

Materials and Methods

Study site and soil sampling

Soil samples were randomly collected from six different locations in Southwest, Nigeria. The first location at the Lagos State University: Faculty of Science, Faculty of Management Science and Lagos State University second gate. The second location at Covenant University: the College of Science and Technology, College of Developmental Studies, Lecture theatre and the Covenant University farm. The third location was Shagamu. All soil samples were collected at a depth of 0 cm to 30 cm using a hand trowel and were transported to the laboratory in sterile polythene bags. Soil samples with no previous history of being cultivated with legumes were obtained from Badagry beach and sterilized at 65°C for 48 h to serve as the control. The composite soil samples from each of the six different locations were then used for the pot experiment in the greenhouse.

Table 1: Number of colonies found on Nutrient agar and Yeast Extract Mannitol Agar.

Locations and dilution factors	NA	YEMA
FMS 10^{-4}	48	84
FMS 10^{-5}	10	24
FOS 10^{-4}	58	60
FOS 10^{-5}	16	13
LG 10^{-4}	101	32
LG 10^{-5}	20	20
SHA 10^{-4}	50	27
SHA 10^{-5}	12	13
CUL 10^{-4}	61	28
CUL 10^{-5}	20	14
CUF 10^{-4}	44	28
CUF 10^{-5}	6	5
Control	0	0

NA: Nutrient Agar; YEMA: Yeast Extract Mannitol Agar; FMS: Faculty of Management Science; FOS: Faculty of Science; LG: LASU (Lagos State University) Gate; SHA: Shagamu; CUL: Covenant University Locations

Table 2: Number of colonies found on Nutrient agar and Yeast extract mannitol agar CFU/g.

Locations and dilution factors	NA (CFU/g)	YEMA (CFU/g)
FMS 10^{-4}	4.8×10^5 (0.48)	8.4×10^5 (0.84)
FMS 10^{-5}	1.0×10^6 (1.0)	2.4×10^6 (2.4)
FOS 10^{-4}	5.8×10^5 (0.58)	6.0×10^5 (0.60)
FOS 10^{-5}	1.6×10^6 (1.6)	1.3×10^6 (1.3)
LG 10^{-4}	1.01×10^6 (1.01)	3.2×10^5 (0.32)
LG 10^{-5}	2.0×10^6 (2.0)	2.0×10^6 (2.0)
SHA 10^{-4}	5.0×10^5 (0.50)	2.7×10^5 (0.27)
SHA 10^{-5}	1.2×10^6 (1.2)	1.3×10^6 (1.3)
CUL 10^{-4}	6.1×10^5 (0.61)	2.8×10^5 (0.28)
CUL 10^{-5}	2.0×10^6 (2.0)	1.4×10^6 (1.4)
CUF 10^{-4}	4.4×10^5 (0.44)	2.8×10^5 (0.28)
CUF 10^{-5}	6.0×10^5 (0.60)	5.0×10^5 (0.50)
Control	0	0

This table shows the converted values of the number of colonies derived from the serial dilution of the various samples to CFU/g.
NA: Nutrient Agar; YEMA: Yeast Extract Mannitol Agar; FMS: Faculty of Management Science; FOS: Faculty of Science; LG: LASU (Lagos State University) Gate; SHA: Shagamu; CUL: Covenant University Locations

Seeds

Legume seeds were obtained from the open market at Ota market.

Media and plant nutrients

Yeast-Extract Mannitol Agar (YEMA), Jensen's nutrient solution and potassium nitrate solution (KNO_3) at 0.05% N were used [14].

Serial dilution of the soil samples

The soil samples were serially diluted with sterile water using dilution factors 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. This was done by filling five labeled test tubes with 9 ml of distilled water, then autoclaved at 121°C. One (1) g of each sample was measured into the first test tube

Table 3: Biochemical tests carried out on the *Rhizobium* isolates from the various locations.

Locations (10^{-5})	Catalase test	Oxidase test	Coagulase test
FMS (Isolate 1)	Negative	Negative	Positive
FMS (Isolate 2)	Negative	Negative	Negative
FOS (Isolate 1)	Negative	Negative	Positive
FOS (Isolate 2)	Negative	Negative	Positive
LG (Isolate 1)	Negative	Negative	Negative
LG (Isolate 2)	Negative	Negative	Negative
SHA (Isolate 1)	Positive	Negative	Positive
SHA (Isolate 2)	Positive	Negative	Negative
CUL (Isolate 1)	Negative	Negative	Positive
CUL (Isolate 2)	Negative	Negative	Negative
CUF (Isolate 1)	Negative	Negative	Negative
CUF (Isolate 2)	Negative	Negative	Negative

It shows the results of the biochemical tests that were carried out on the various isolates for identification of the isolates. For catalase test, most of the organisms were negative with ratio 2:10, for oxidase, most of the organisms were negative with ratio 0:12 and for coagulase, most of the organisms were negative with ratio 4:8.

NA: Nutrient Agar; YEMA: Yeast Extract Mannitol Agar; FMS: Faculty of Management Science; FOS: Faculty of Science; LG: LASU (Lagos State University) Gate; SHA: Shagamu; CUL: Covenant University Locations

Table 4: Morphological characteristics of *Rhizobium* isolate from the various locations.

Locations	Cell shape	Gram staining	Cell motility
FMS (Isolate 1)	Rod	Gram negative	Positive
FMS (Isolate 2)	Rod	Gram negative	Negative
FOS (Isolate 1)	Rod	Gram negative	Negative
FOS (Isolate 2)	Rod	Gram negative	Negative
LG (Isolate 1)	Rod	Gram negative	Positive
LG (Isolate 2)	Rod	Gram negative	Positive
SHA (Isolate 1)	Rod	Gram negative	Negative
SHA (Isolate 2)	Rod	Gram negative	Negative
CUL (Isolate 1)	Rod	Gram negative	Positive
CUL (Isolate 2)	Rod	Gram negative	Positive
CUF (Isolate 1)	Rod	Gram negative	Negative
CUF (Isolate 2)	Rod	Gram negative	Positive

NA: Nutrient Agar; YEMA: Yeast Extract Mannitol Agar; FMS: Faculty of Management Science; FOS: Faculty of Science; LG: LASU (Lagos State University) Gate; SHA: Shagamu; CUL: Covenant University Locations

labeled 10^{-1} then mixed properly. 1 ml of the mixture was dispensed into the next test tube labeled 10^{-2} and same was done for test tubes 10^{-3}, 10^{-4} and 10^{-5}.

Approximately, 0.1 ml sample of dilutions 10^{-4} and 10^{-5} of each sample were inoculated onto cooled NA and YEMA using the pour plate method. The inoculated plates were incubated at room temperature for 48 h in an improvised carton with holes by the sides to allow oxygen entry; this was to serve as the incubator. Thereafter, the colonies were counted using a colony counter devise.

Nodulation pot experiment

The composite soil samples were tested with soybean seeds (*G. max*) to ascertain the potential of the crop legume to nodulate with promiscuous indigenous *bradyrhizobium*. Sixteen plastic pots of 20 mm diameter were used to cultivate the legume species replicated twice and a total of four control soil samples were used replicated twice and arranged on a platform in the greenhouse. Two seeds of soybeans were sown aseptically in each pot with sterile forceps. Each pot was watered with sterile water, two of the control pots received Jensen's nutrient solution and the other two received 0.05% KNO_3 solution for 8 weeks [3].

Results

All isolates were rods and Gram negative and most of the colonies observed were all round, all mucoid, 58.3% of the colonies were creamy, 16.67% were yellowish and 16.67% were white. This findings was corroborated by Bantu et al. [15], where the colonies seen were mostly mucoid (Tables 1-5).

Discussion and Conclusion

The presence of *Rhizobium* in the soil is necessary for atmospheric nitrogen fixation *via* the root nodules. *Rhizobium* converts atmospheric nitrogen to utilizable forms of nitrogen for the plants for growth and development. Although, favourable climatic and edaphic factors encouraged the establishment of *G. max* in the tropical soils investigated. Crop legumes are selective for *Bradyrhizobium* species in tropical soils as microsymbiont due to the innate trait for alkalinophilic properties of *Bradyrhizobium sp.* However, promiscuity among certain *Rhizobium sp.* has led to few cases of Soybean nodulated by *Rhizobium sp.* [16,17].

It was evident that the indigenous *rhizobia* populations were adequate for nodulation of the legume roots as well as the fact that they displayed their potential to infect legume roots. Tables 1-3 contain the number of colonies derived from NA and YEMA after conversion to the CFU/g unit. The values in parentheses are the number of colonies in CFU/ml that were converted to the 10^{-6} factor for the plotting of the bar chart. The bar chart in Figure 1 clearly showed that 41.6% of YEMA cultures had a lower microbial load compared to NA which had 58.4%. Nutrient agar supports the growth of various microorganisms, this is why it has a higher percentage of microorganisms and it gave a more diverse result and helps with further investigation which is in agreement with previous report [18].

Biochemical tests were carried out on the various isolates from

Table 5: Morphological characteristics of *Rhizobium* isolates from the various locations.

Locations (10^{-5})	Nature of colony	Colour of colony
FMS (Isolate 1)	Translucent, mucoid	Creamy
FMS (Isolate 2)	Translucent, mucoid	Creamy
FOS (Isolate 1)	Opaque, mucoid	Creamy
FOS (Isolate 2)	Opaque, mucoid	Creamy
LG (Isolate 1)	Opaque, mucoid	White
LG (Isolate 2)	Translucent, mucoid	White
SHA (Isolate 1)	Opaque, mucoid	Light cream
SHA (Isolate 2)	Transparent, mucoid	Transparent
CUL (Isolate 1)	Opaque, mucoid	Yellowish
CUL (Isolate 2)	Opaque, mucoid	Yellowish
CUF (Isolate 1)	Opaque, mucoid	Creamy
CUF (Isolate 2)	Opaque, mucoid	Creamy

NA: Nutrient Agar; YEMA: Yeast Extract Mannitol Agar; FMS: Faculty of Management Science; FOS: Faculty of Science; LG: LASU (Lagos State University) Gate; SHA: Shagamu; CUL: Covenant University Locations; CUF: Covenant University Farm

the YEMA plates where 10^{-5} colonies were sub-cultured from, to determine their biochemical characteristics and morphology, tests were also carried out on the isolates to determine their morphological characteristic features and most of the colonies observed were all round, all mucoid, 58.3% of the colonies were creamy, 16.67% were yellowish and 16.67% were white (Tables 4 and 5). This corroborated the previous findings of Bantu et al. [15]. The morphological and biochemical characteristics of these isolates suggested they were *Bradyrhizobium* species.

Conclusion

Rhizobium is very relevant to plant growth and maintenance of soil fertility due to its ability to convert atmospheric nitrogen to ammonia for its utilization by plants. The introduction of effective and persistent bradyrhizobia species into soybean farms would enhance soybean production as well as encourage sustainable agricultural practice. The use of biological fertilizer in the form of bradyrhizobia inoculants would serve as a low-input and cheap bio-fertilizer input into agriculture, thus preventing the use of scarce resources in procurement of chemical fertilizers as well as impacting positively on adjacent farmlands.

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