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Phenotypic and Temperature Tolerance Variations in Bacterial Endophytes Isolated from Cowpea (*Vigna unguiculata* L. Walp) Nodules Recovered from Smallholder Farms in Northern Nigeria

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Abstract

The significance of cowpea (*Vigna unguiculata* L. Walp) as nutritional, economic and a soil fertility promoter had already been elucidated by many researchers. The present work was aimed at isolation and characterization of rhizobial and non-rhizobial endophytes of cowpea nodules recovered from smallholder farms in northern Nigeria. The study soils were analyzed and found to have varying physical and chemical characteristics. Nine (75 %) of the soil samples were sandy loam while the others included sand loam, sand and clay loam each. The pH (water) of the soils ranged from moderately acidic to neutral. The fertility status of the soils in respect to total nitrogen (%), (phosphorus mg kg^{-1}) potassium (Exc K), and organic carbon (%) varied among sites. Each of the 40 nodular endophytes was isolated using yeast mannitol agar and phenotypically characterized using 14 assays (5-growth, 4-sugar fementation, 5-temperature courses). In all, 35 % of the isolates tested positive for the 14 assays. All isolates grew in nutrient agar, King's B agar; and 30 °C and 40 °C. Hierarchical Cluster Analysis (HCA) indicated diversity and similarities among isolates from same and different localities. Soil origin, its features or conditions did not appear to determine the phenotypes of the bacterial isolates. Growth of some of the isolates on *Pseudomonas* selective agar suggests that bacteria resident in cowpea nodules included at least rhizobia and pseudomonads flora.

Keywords: Legumes, *Pseudomonas*, Rhizobia, Soil, Sub-saharan Africa

Introduction

It is now known that plants harbour epiphytic or endophytic communities of bacteria that colonize almost all tissues (Venieraki *et al.,* 2016), with many isolated from legume plants such as alfalfa, clover, and soybean (*Stajković et al.,* 2009). Valid species definitions require phenotypic description, with growth phenotypes allowing microbiologists to describe and thereby differentiate cells. Bacteria are able to communicate with each other and 'sense' their environment in a population density dependent mechanism known as quorum sensing (QS) (Götz-Rösch *et al.,* 2015). QS has been indicated among Gram-negative bacteria which are frequent colonizers of rhizospheres (Dourado *et al.,* 2013; Sanchez-Contreras *et al.,* 2007). QS systems are often responsible for the regulation of various phenotypic characteristics in numerous plant associated bacteria (Alavi *et al.,* 2013).

Cowpea cultivated by smallholders in northern Nigeria has the potentials to sources indigenous nodular endophytes in addition to alleviating hunger and poverty in rural populace. This research was aimed at phenotypic characterization of bacterial endophytes isolated from cowpea nodules recovered from smallholder farms in some parts of northern Nigeria.

Materials and Methods

Sites of Nodular Recovery

Nodules were recovered according to the methods of Somasegaran and Hoben (1985) from the locations in Adamawa, Bauchi, Gombe and Kano states, shown in Table I. All areas are in the dry sub humid agro ecological zones of Nigeria (Chude *et al.,* 2012).

Soil Sampling

Soil sample collections in all the study areas were conducted as described by Abaidoo *et al*., (2007). Four replicate quadrant plots (100 m^2) were determined, with six soil cores per quadrant randomly removed using a 6 cm diameter corer, to a depth of 20 cm. The six soil core samples were combined into a composite sample (replicate); air-dried on an open bench in the laboratory for 72 h before physical and chemical analyses.

Determination of Physical and Chemical Characteristics of Soils

Textural class of the soils was determined using the hydrometer method (Bouyoucos, 1951). The pH was determined according to Bates (1954) and IITA (1979). Twenty grams of soil were added to 20 ml of sterile distilled water and the pH was measured. Determination of electrical conductivity of the soil samples was carried out as described in Hanna (1964) and Rhoades, (1982), and electrical conductivity of the supernatant measured using EC Meter. Percentage moisture content of the soils from the study sites was determined was also determined (ITTA, 1982). The procedure of Walkley and Black (1934) was used to determine the organic carbon (OC) content. Phosphorous and Potassium (K) contents were measured using the Bray I method (ICARDA, 2013). Determination of total nitrogen was by Kjeldahl method as described by IITA (1982).

Nodule Recovery for Endophytic Isolation

Nodules were recovered according to protocols described by Somasegaran and Hoben (1985) from cowpea plants during vegetative stage from different smallholder farms in the study locations (Table I). A sturdy spade was used to excavate the whole root system around a radius of about 20 cm and a depth of up to 50 cm of the plants, taking care not to detach the nodules. The plants and rhizosphere soil were transported to the laboratory in clean paper bags. Then the soil was gently washed (Figure I), and the medium sized pinkish nodules were carefully excised using sterile lancet and forceps with about 2 cm root tissue still attached to avoid injury to the nodule tissue. Detached and connected cowpea nodules were not selected for endophytic bacterial isolations.The recovered nodules were stored at 4 ºC in screw top jars containing dessicated silica gels until isolation.

Isolation of Endophytic Bacteria

Isolation of endophytes was according to Somasegaran and Hoben (1985). The desiccated nodules were rehydrated by immersion in clean beaker of cool water and leaving the container in the refrigerator to imbibe overnight. The nodules were then washed thoroughly to remove soil. From this point, all the subsequent stages were carried out gnotobiotically, using sterile instruments and media in a laminar flow cabinet to ensure authenticity of isolates as endophytes. Surface sterilization was conducted by immersing intact, undamaged, nodules in 95 % ethanol for 5-10 seconds to break surface tension and to remove air bubbles from nodule tissues. Then the nodules were transferred to 3 % solution of sodium hypochlorite for 5 min, and then rinsed in 10 changes of sterile water.

Surface sterilized nodules were crushed with a pair of sterile blunt-tipped forceps in a large drop of sterile water in a Petri dish. One loopful of the nodule suspension was streaked on a Yeast-Mannitol Agar (YMA) plate and incubated at 28 ºC for 3 days. Single colonies of varying cultural characteristics were re streaked until pure cultures were obtained. Single colonies were picked up and were re-streaked on YEMA plates for obtaining pure cultures. Pure cultures were stored on YMA slants in 30 ml screw cap bottles pending phenotypic analyses.

Colony Characteristics of the Bacterial Isolates

Colour and size of the colonies were assessed as described by Kimiti and Odee (2010) at 3 days after incubation at 28 °C. The sizes of the colonies formed were recorded as small for colonies less that 2 mm in diameter; medium for colonies 2 mm to 3 mm; and large for colonies greater than 3 mm in diameter. The colour of the colonies was assessed by visual examination. The texture was first assessed by visual examination and confirmed by manipulation using sterile wooden toothpick (Tesco, UK); wet colonies were watery and easy to pick a small quantity; dry colonies required some scraping on the media to pick; mucoidal colonies were copious and needed no toothpick manipulation for confirmation; while sticky colonies were gummy, clump up therefore hard to pick with the toothpick.

Nutrient Utilization and Sugar Fermentation

Ten µL of overnight YMB culture were innoculated into each petri dish containing nutrient agar (NA), Luria Bertani agar (LBA), *Pseudomonas* selective agar (PSA, *Pseudomonas* selective agar plate with cetrimide, fusidin, and cephaloridine, CFC supplements, Oxoid, UK) and sodium citrate modified agar (SCA, sodium citrate substitutes mannitol). For sugar fermentation assays, Mannitol in YMA was substituted with arabinose, galactose, lactose, and sucrose (Sambrook *et al.,* 1989; Robertson *et al.*, 2013). All procedures were replicated four times and incubated at 28 °C. A positive result was recorded if growth was observed after 3 days and negative if there was no growth (Sambrook *et al.,* 1989; Robertson *et al.*, 2013).

Temperature Tolerance

Temperature sensitivity was assessed using the procedure described by Robertson *et al.* (2013). Ten microliters of 10^{-1} diluted overnight YMB culture was drop inoculated into YMA plates and incubated at a temperature of 10 °C, 20 °C, 30 °C, 40 °C and 50 °C for 3 days, control plates were incubated at 28 °C for the same duration. A positive result was recorded if growth was observed and a negative if there was no growth.

Statistical Analyses

Temperature tolerance was analysed by Hierarchical Cluster Analysis (HCA) using the Ward method without any weighting of factors (phenotypes, i.e. each factor were treated equally, JMP 7.0).

Results

Characteristics and Fertility Status of Soils

Results of the soil analysis showed similarities and variations among the sites. Classification of fertility status based on physical and chemical analyses of the soil samples collected from the nodule recovery farms are shown in Table II.

About 75 % of the soils were sandy loam textured. Site C, K and G were sand loam, sand and clay loam, respectively. Site G had the highest moisture content of 3.45 %, followed by J with 2.74 %. While followed by B and K at 0.67 %, and then, C and H have the lowest moisture contents of 0.33 % each. Electrical conductivities of the soils vary from 0.02 and 0.7.

Endophytic Bacterial Isolation from Recovered Nodules

Pure cultures of isolates were obtained after multiple subculturing of initial isolations under gnotobiotic conditions. A total of 40 isolates were selected from the 12 locations for the pure cultures as shown in Table III. Random colonies were subsequently selected based on single colony texture, colour, and size. A single colony was selected from H due to its homogeneity.

Colony Characteristics of the Bacterial Isolates

Colony characteristics were assessed three days after incubation of a 10µl culture smear on YMA. Individual Colonies were classified as small (<2 mm); medium (2-3 mm) and large (>3 mm) in

diameter. Values of 35 %, 37.5 % and 25 % of the endophytes were initially isolated from small, medium and big colonies, respectively. The most common texture of isolates, 40 % was the watery form, followed by sticky (25 %). Total of 22.5% of the isolates were mucoidal, while the least proportion was the dry texture form with 12.5 %. Visual colour assessments on YMA indicated that 52.5 % were white, 22.5 % cream, 17.5 % yellow, and only 7.5 % were brown.

Nutrient Utilization and Sugar Fermentation

At three days after inoculation, most isolates (97.5 %) tested positive for growth in both KB and nutrient agar; 72.5 % tested positive for growth in LBA, while 70 % isolates were positive in PSA and SCA. In sugar fermentation assays, positive results were obtained in sucrose (95 %), arabinose (92.5 %), lactose (90 %). and galactose (77.5 %).

Temperature Tolerance

All isolates tested positive for growth in plates incubated at 30 °C and 40 °C for 72 hours. Ratios of 36/40, 4/40, and 12/40 negative growths were observed in 10 °C, 20 °C, and 50 °C respectively. The constellation plot (Figure 2) indicates that most of the isolates were in the temperature tolerant groups I and II.

Group I members grew in all the temperatures tested (10 °C, 20 °C, 30 °C, 40 °C and 50 °C). Group II isolates (14F, 16F, 19G and 24G) were sensitive to 50 °C only. Group III (1A, 5B, 6B, 8C, 22G, 25G, 26H and 39L) are the endophytes that grew at 20 °C, 30 °C, 40 °C, which include the two isolates of site B and the single isolate from site H. Group IV consists of the endophytes (3A, 4A, 37L and 38L) with the least range of temperature tolerance, with positive growth for the four isolates recorded at 30 °C and 40 °C only. It was also observed that warmer temperatures up to 40 °C stimulated more growth than the control (28 °C).

Phenotypic differences of the isolates according to colony characteristics, growth in media, sugar fermentation and temperature tolerance in the fourteen assays conducted are shown in Table IV.

Discussion

The focus for this research was isolation of indigenous endophytes. Sampling site selection was simply a reflection of localities that cultivated cowpea. An attempt was made to focus mainly on areas of Kano state which the author was familiar, and in which interaction with farmers had been established prior to the research, purposely to rule out the chances of previous bio fertilizer application.

The results of the soil analyses indicated that cowpea could thrive in sand, as well as, in clay loam confirmed that the crop is versatile, adapted to variety of soils. Soil pH (water) in the 12 sites ranged from moderately acidic to neutral, this is, in contrast to Abaidoo *et al.,* (2007) whose 12 sites were acidic to moderately acidic (4.4 to 6), and Kapembwa *et al.,* (2016) who had 4.8 to 6.0, but both supported legume crop in Africa. The organic Carbon contents of the study sites ranged from very low (I, J and H) to moderate (G) this is also in contrast to Abaidoo *et al.,* (2007) whose sites ranged from very low to low organic carbon contents. Kapembwa *et al.,* (2016)

isolated rhizobia in soils with organic carbon contents below critical levels. Nitrogen contents of soil had been found to be significant in nodulation studies, however even in sites B C and K, where the N levels were high; nodulation was successful on the excavated cowpea plants. Nodulation also did not seem impaired by very low N contents. Kapembwa *et al.,* (2016) isolated rhizobia in three sites in Zambia with N contents 67 % lower than critical value. Phosphorus contents ranged from low (J, H and F) to high (A and B), similar to Abaidoo *et al.,* (2007) but not Kapembwa *et al.,* (2016). By Chude *et al.,* (2012) soil fertility status, some of present study sites had high potassium content.

Endophytic isolation was carried out from medium sized healthy intact nodules. Detached and connected (fused) cowpea nodules were not selected for endophytic bacterial isolations even if they appeared healthy and intact, because the point of detachment is an avenue for contaminating microbes to gain entry into the nodule. Likewise, fused nodules may contain cracks and injury that may not be apparent on examination and tended to be large and their crevices would always hinder successful surface sterilization.

Characterization results show that the cowpea nodules host bacteria with diverse phenotypic variations. Similar results were reported by Rajendran *et al.,* (2012) who isolated 17 different endophytes from *Trigonella foenum-graecum* (fenugreek) nodules; Aserse *et al.,* (2013) isolated non rhizobial endophytes from the root nodules of leguminous plants *Crotalaria incana* and *Phaseolus vulgaris.* Other researchers worked on endophytes in non leguminous crops, for example, Arunachalam and Gayathri (2010) isolated endophytic bacteria from the medicinal plant *Andrographis paniculata*; Alavi *et al.* (2013) reported that *Stenotrophomonas maltophilia* can be both seed-borne and occur as an endophyte in Brassicaceae oilseed rape plant; *Asif et al.* (2016) isolated an endophytic *Pseudomonas putida* from mango orchard and Arthee and Marimuthu, (2017) isolated 22 sugarcane endophytes, of which five were identified as *Burkholderia* Spp.

The variations in the morphology of the isolates in this study agree with the findings of Abdelnaby *et al.* (2015) who isolated a total of 30 rhizobia from root nodules of two cultivars of Cowpea plants, and found that each nodule was hosting two types of bacteria. One type has a colony similar to fast-growing rhizobia, producing large gum and developed colonies within 2-3 days. Whereas, the second type showed characteristics similar to slow-growing rhizobia-like, that produces raised, shiny, creamy and small colonies (1 mm after more than 5 days of incubation). Adiguzel *et al.* (2010) isolated rhizobia from wild vetch in Turkey that had cream and orange mucoidal colonies. A species of *Burkholderia* characterized by Arthee and Marimuthu (2017) were half dry, half mucoidal, creamy white and dull yellow in colour. Similar results were also reported by Kapembwa *et al.* (2016) who, in addition to cream, white, yellow, and transparent, also found 3 % pink colonies.

The ability of endophytes to grow in different media such as NA LBA, PSA, and utilize different sugars (lactose the most unfermented) as carbon sources shown in this work has also been reported by recent findings on cowpea nodular and endophytes (Bhatt *et al.,* 2013; Shrivastava, 2013**;** Asif *et al.,* 2016;

Hamza and Alebejo, 2017). Also, similar results were reported by Dixit *et al*. (2014) and Elzanaty *et al.* (2015). These works also discovered variations in citrate utilization among the isolates as reported in this research.

Temperature profiles indicated that isolates were adapted to wide range of tempeartures. Temperatures in regions of isolations of these endophytes would drop as low as 10 °C during the harmattan season and can reach up to upper 30s in the summer. However, there were more negatives (10 %) in 10 °C than in 50 °C. This is likely due to the fact that the isolates are more adapted to higher temperatures than lower temperatures. Abdelnaby *et al.* (2015) reported that the growth temperature for most *Bradyrhizobium* spp. from different legumes was below 38 °C. Bhatt *et al.,* (2013) did not see growth of root nodule bacteria of Mung Bean (*Vigna radiate*) at 40 °C. Hierarchical Clustering of the isolates did not differentiate isolates of the hotter north eastern Nigeria than those from the slightly cooler north western Nigeria.

Conclusions

Growth in Yeast mannitol agar, a selective medium for the isolation of rhizobia, and the growth of some of isolates in *Pseudomonas* selective media confirm that the cowpea nodule is a niche for an array of bacterial endophytes. Isolate 16F, tested negative for all the growth assays, while isolates 10D and 35K were positive for 3 and 5 of the assays, at 3 days after inoculation, indicating they were slow growing rhizobia, commonly associated with tropical legumes. The ability of the nodular endophytes to utilize a wide range of nutrients and tolerate 10 °C and 50 °C temperatures, render them suitable for biotechnological manipulations.

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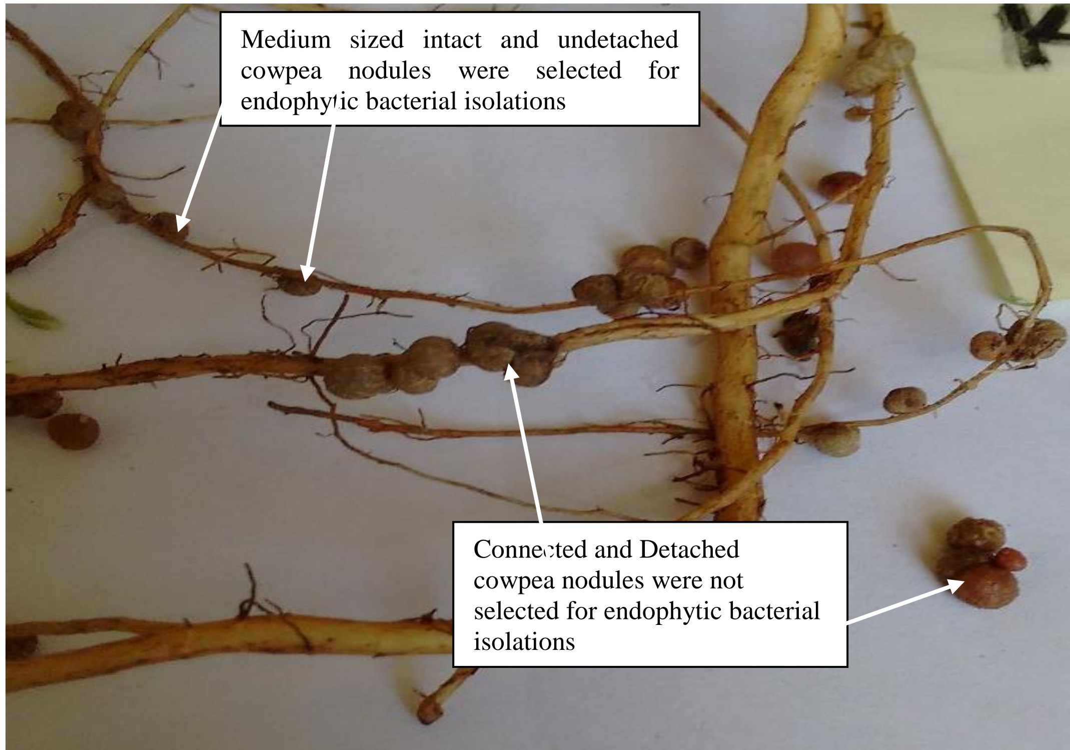

Figure 1: Washed Cowpea Nodules

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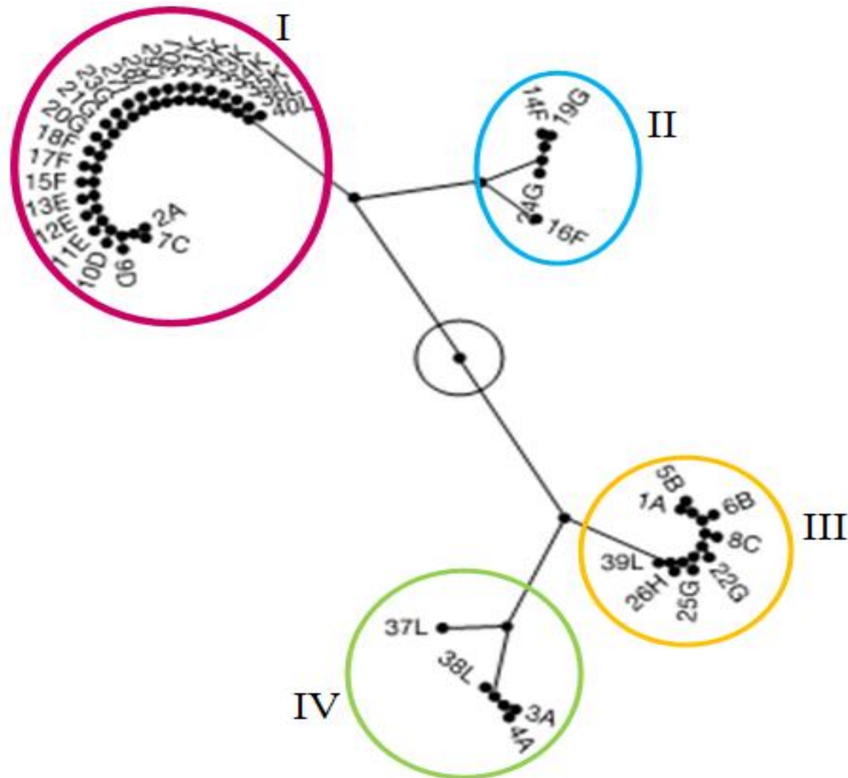

Figure 2: Isolates Groupings in Response to Temperature Tolerance

Table I: Study Site for Nodule Recovery

Site	**Description**	**GPS Location**
A	Kano 1	11.67^{0} 85′ N; 8.26^{0} 32′ E
B	Bauchi	10.30^{0} 16′ N; 9.84^{0} 18′ E
C	Gombe	09.96^{0} 22′ N;11.16^{0} 50′ E
D	Kano 2	11.98^{0} 82′ N; 8.35^{0} 96′ E
E	Kano 3	12.19^{0} 25′ N; 8.63^{0} 01′ E
F	Kano 4	11.98^{0} 02′ N; 8.45^{0} 71′ E
G	Adamawa 1	09.33^{0} 80′ N;12.23^{0} 84′ E
H	Kano 5	12.09^{0} 90′ N: 8.50^{0} 84′ E
I	Kano 6	11.98^{0} 72′ N; 8.43^{0} 22′ E
J	Kano 7	11.79^{0} 79′ N; 8.02^{0} 12′ E
K	Adamawa 2	09.20^{0} 74′ N;12.48^{0} 84′ E
L	Kano 8	12.11^{0} 07′ N; 8.75^{0} 68′ E

Table II: Soil Characteristics and Fertility Status.

Site	TN (%)	P (mg/kg)	K (cmol/kg)	O/C (%)	pH (H_2O)	EC (ds/m)	M/C (%)	T/C
A (Kano 1)	MH	H	VH	L	N	0.12	1.01	sandy loam
B (Bauchi)	H	H	M	L	N	0.06	0.67	sandy loam
C (Gombe)	H	M	L	L	SA	0.03	0.33	Sand loam
D (Kano 2)	ML	M	M	L	SA	0.19	0.88	sandy loam
E (Kano 3)	VL	M	L	L	SA	0.13	1.01	sandy loam
F (Kano 4)	ML	L	M	L	SA	0.06	2.04	sandy loam
G (Adamawa1)	MH	M	VH	M	SA	0.18	3.45	clay loam
H (Kano 5)	VL	L	L	VL	SA	0.05	0.33	sandy loam
I (Kano 6)	VL	M	H	VL	N	0.15	0.81	sandy loam
J (Kano 7)	ML	L	L	VL	MA	0.02	2.74	sandy loam
K (Adamawa2)	L	M	L	L	N	0.04	0.67	sand
L (Kano 8)	L	M	L	L	MA	0.12	0.80	sandy loam

TN-Total Nitrogen **P-**Phosphorus **K-**Potassium **O/C-** Organic Carbon **EC-**Electrical Conductivity **M/C-** Moisture Content **T/C-** Textural Class; **H**-High **MH**-Moderately High **M**-Moderate **ML**-Moderately Low **L**- Low **VL**-Very Low **N**-Neutral **SA**-Slightly Acidic **MA**- Moderately Acidic

Table III: Isolates Designated Nomenclature based on Study Locations

Location of Nodule Recovery	Isolates and Designated Nomenclature
1. Kano 1	1A, 2A, 3A and 4A
2. Bauchi	5B and 6B
3. Gombe	7C and 8C
4. Kano 2	9D and 10D
5. Kano 3	11E, 12E, and 13E
6. Kano 4	14F, 15F, 16F, 17F and 18F
7. Adamawa 1	19G, 20G, 21G, 22G, 23G, 24G and 25G
8. Kano 5	26H
9. Kano 6	27I and 28I
10. Kano 7	29J and 30J
11. Adamawa 2	31K, 32K, 33K, 34K and 35K
12. Kano 8	36L, 37L, 38L,39L and 40L

Table IV: Phenotypic Variations of Cowpea Nodular Endophytes. Note: Almost each isolate is unique in at least one of four respects.

Isolate	Colony Characteristics	Nutrient Utilisation	Sugar Fermentation	Temperature Tolerance (°C)
1A	Watery/White/Small	NA, KBA, LBA, SCA	Arab, Lac, Suc	20 , 30, 40
2A	Watery/White/Medium	NA, KBA, PSA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
3A	Watery /Brown/ Large	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	30, 40
4A	Watery /Yellow/ Large	NA, KBA, SCA	Arab, Gal, Lac, Suc	30, 40
5B	Sticky/Cream/Small	NA, KBA, SCA	Arab, Lac, Suc	20 , 30, 40
6B	Dry/Yellow/Medium	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	20 , 30, 40
7C	Mucoid /Yellow/ Small	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	20 , 30, 40
8C	Mucoid /Yellow/ Medium	NA, KBA, PSA, SCA	Arab, Gal, Suc	20 , 30, 40
9D	Watery/White/Small	NA, KBA, LBA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
10D	Watery/White/Medium	NA, KBA	Lac	10, 20, 30, 40,

Isolate	Colony Characteristics	Nutrient Utilisation	Sugar Fermentation	Temperature Tolerance (°C)
				50
11E	Watery/White/Medium	NA, KBA, LBA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
12E	Sticky/White/Medium	NA, KBA, LBA, PSA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
13E	Sticky/Brown/Small	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
14F	Mucoid /White/ Large	NA, KBA, LBA, PSA	Arab, Lac, Suc	20, 30, 40, 50
15F	Mucoid /White/ Large	NA, KBA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
16F	Watery/White/Medium			20, 30, 40, 50
17F	Watery/White/Small	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
18F	Watery/White/Small	NA, KBA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
19G	Watery/White/Large	NA, KBA, LBA, PSA, SCA	Arab, Lac, Suc	20, 30, 40, 50
20G	Dry/White/Large	NA, KBA, LBA, PSA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
21G	Watery/Cream/Small	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
22G	Sticky/Cream/Medium	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	20 , 30, 40
23G	Watery/Cream/Medium	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
24G	Mucoid /Cream/ Small	NA, KBA,LBA, PSA, SCA	Arab, Gal, Lac, Suc	20, 30, 40, 50
25G	Mucoid /Cream/ Small	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	20 , 30, 40
26H	Watery/White/Small	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	20 , 30, 40
27I	Mucoid /White/ Medium	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
28I	Mucoid /White/ Small	NA, KBA, LBA, PSA,	Arab, Lac, Suc	10, 20, 30, 40,

Isolate	Colony Characteristics	Nutrient Utilisation	Sugar Fermentation	Temperature Tolerance (°C)
		SCA		50
29J	Watery/Yellow/Small	NA, KBA, LBA, PSA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
30J	Sticky/White/Medium	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
31K	Sticky/White/Large	NA, KBA, LBA, , SCA	Gal, Lac, Suc	10, 20, 30, 40, 50
32K	Sticky/Cream/Large	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
33K	Sticky/Cream/Medium	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50
34K	Sticky/Brown/Medium	NA, KBA, LBA, SCA	Arab, Lac, Suc	10, 20, 30, 40, 50
35K	Dry/Yellow/Small	NA, KBA	Arab, Gal, Suc	10, 20, 30, 40, 50
36L	Dry/Yellow/Medium	NA, KBA, LBA, PSA	Arab, Gal, Suc	10, 20, 30, 40, 50
37L	Mucoid /White/ Medium	NA, KBA, PSA	Arab, Gal, Lac, Suc	20 , 30
38L	Dry/White/Medium	NA, KBA, PSA, SCA	Arab, Lac, Suc	20 , 30
39L	Sticky/White/Small	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	20 , 30, 40
40L	Watery/Cream/Small	NA, KBA, LBA, PSA, SCA	Arab, Gal, Lac, Suc	10, 20, 30, 40, 50

Foot Note: NA (Nutrient Agar), KB (King's B Agar), LB (Luria Bertani Agar), PSA (*Pseudomonas* Selective Agar), SCA (Sodium Citrate modified Agar), Arab (Arabinose), Gal (Galactose), Lac (Lactose), Suc (Sucrose)