



Research Article

Biofilm Formation Capability of Cowpea Nodular Bacterial Isolates in Static Microcosms

Rabiu HM^{*1,2}, Deeni YY², Kawo AH³, Kabir K², Bamanga R², Spiers AJ²

¹Department of Biological Sciences, Bayero University Kano, Nigeria

²Simbios Centre, School of Science, Engineering and Technology, Abertay University, Dundee UK

³Department of Microbiology, Bayero University Kano, Nigeria

*Corresponding author: hmrabiu.bio@buk.edu.ng, doi.org/10.55639/607.4757

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ABSTRACT

Bacteria are known to form microbially derived sessile communities that are irreversibly attached to biotic or abiotic surfaces known as biofilms. Biofilm formation is an adaptive strategy for the successful colonization of plant surfaces. Forty bacterial isolates of cowpea nodules from 12 smallholder farms in Northern Nigeria were evaluated for biofilm growth, attachment and strength in static microcosms. Replicate King's B (KB) microcosms ($n = 8$) were incubated statically for 7 days before assessments. Growth and attachment were determined by measuring optical density (OD_{600nm}) and absorbance (A_{570 nm}) respectively. Strength (grams) was determined by maximum deformation mass (MDM) assay using glass balls (mean weight of 0.0115g). A significant MDM was recorded when more than one replicate retained at least one ball for at least 5 seconds. One-way Analysis of Variance (ANOVA) was used to determine significant mean differences ($p < 0.05$). Similarities and variations in biofilm formation were assessed by Hierarchical Cluster Analysis (HCA) using the Ward method. Half of the isolates showed good biofilm growth and attachment. Twelve of the 40 isolates showed various levels of strength. Isolates 16F and 39L were rated well in all three biofilm parameters. There were significant differences in the mean values of the assessed biofilm parameters among the isolates. Mean growth ranged between 1.493 (Isolate 32K) and 2.101 OD₆₀₀ (Isolate 19G). Mean attachment levels (A₅₇₀) ranged from 0.084 (Isolate 9D) to 1.543 (Isolate 30K). Only isolate 2A had significantly higher biofilm strength (2.412g) than the rest of the isolates. HCA analysis indicated similarities as well as variations in biofilm formation by isolates within and between sites. Isolates from all the study sites were positive for at least one of the tested biofilm parameters. The ability of the isolates to form biofilms indicated that some of them could be suitable for biotechnological applications such as in biofertilizer production

Corresponding author Rabiu HM, Email: hmrabiu.bio@buk.edu.ng
Department of Biological Sciences, Bayero University Kano, Nigeria

INTRODUCTION

Bacteria are widely distributed and abundant in the environment (Nemergut *et al.*, 2011; Zhang *et al.*, 2013). Bacterial floras are dispersed within the soil, often attached to soil particles and roots of plants (Wright, 2010; Goldfarb *et al.*, 2011). Biofilms are defined as microbially derived sessile communities initiated when free-floating bacteria (planktonic) suddenly change to a sessile lifestyle to form characterized cells that are irreversibly attached to biotic or abiotic surfaces (Moreau-Marquis *et al.*, 2008; Gu and Ren, 2014). Within a biofilm, bacteria communicate with each other by the production of chemotactic particles or pheromones through quorum sensing (Hassan *et al.*, 2011). Rhizosphere bacterial populations are hotspots for microbial interaction and biofilm formation leading to root colonization (Sereviratne *et al.*, 2011). Experimental models with abiotic surfaces are useful for the initial characterization of the structure of rhizobial biofilms, and of the necessary conditions for biofilm formation (Rinaudi and Giordano, 2010). Bacterial cultures growing in experimental microcosms (e.g. glass vials containing liquid growth medium) can form biofilms at the air-liquid (A-L) interface. These biofilms growing on the liquid surface or at the meniscus can be divided into different classes based on their physical strength, resilience and attachment (Spiers *et al.*, 2006).

Cowpea (*Vigna unguiculata* L. Walp): family Fabaceae is a major component of traditional cropping systems in northern Nigeria. The average yield of cowpea in Nigeria was about 577 kg/ha, the production area was 3.7 million hectares and the total production was 2.13 million tons in 2014 (FAO, 2015). It improves soil fertility through biological nitrogen fixation with rhizobia, suppresses weeds, and

is a key source of proteins (Kimiti and Odee, 2010).

This work aimed to determine whether cowpea nodular bacterial isolates produced biofilms on a medium other than plant tissues which is an indication that they might be used in bioremediation and other biotechnology applications in agricultural and other industries. Biotechnological applications of cowpea endophytes would add significance to an already highly valuable crop cultivated by smallholder farmers in northern Nigeria. This would be a novel finding as there is no previous similar work on bacteria isolated from cowpea nodules to the best of the researchers' knowledge.

METHODS

Cowpea Nodule Collection and Isolation of nodular bacteria

Cowpea nodules collection from 12 smallholder farms in northern Nigeria and isolation of nodular bacteria were carried out according to Somasegaran and Hoben (1985) during the 2015 rainy season and stored over silica gel until isolation. The desiccated nodules were rehydrated by immersion in a clean beaker of cool water and leaving the container in the refrigerator to imbibe overnight. The nodules were then washed thoroughly to remove the soil. From this point, all the subsequent stages were carried out gnotobiotically, using sterile instruments and media in a laminar flow cabinet to ensure the 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 remove air bubbles from nodule tissues. Then the nodules were transferred to a 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 Nutrient Agar (NA) plate and incubated at 28 °C for 7 days. Single colonies of varying cultural characteristics were re-streaked until pure cultures were obtained.

Quantitative Assessment of Biofilm-Formation in Static Microcosms

To investigate growth, biofilm strength and attachment levels in static microcosms, combined biofilm assays were used (Robertson *et al.*, 2013). Replicate KB microcosms ($n = 8$) were incubated statically for 7 days before the assessment.

Evaluation of the growth of isolates in static microcosms

Microcosms containing the 7 days culture for each of the 40 selected isolates were each emptied into another sterile vial and vortexed for the 30s. Growth of each isolate was determined by measuring the optical density (OD₆₀₀) using a Spectronic Helios Epsilon spectrophotometer (Thermo Fisher Scientific, UK) with 10 mm optical path cuvettes according to Spiers *et al.* (2003)

Evaluation of Attachment of Isolates in Static Microcosms

The original emptied microcosm vials that contained the 7 days cultures were washed three times in distilled water with vigorous shaking. They were then stained with 1 ml of 0.05 % (w/v) Crystal violet for 2min before washing them three times again in water. The stain was eluted by shaking with 5ml of 95 % ethanol for 2h, and the level of attachment of this solution was determined by measuring the absorbance (A) using spectrophotometry at 570 nm (Spiers *et al.*, 2003).

Evaluation of Strength of Biofilm formed by Isolates

The strength (grams) of the biofilm formed by each isolate was determined by maximum deformation mass (MDM) assay (Ude *et al.*, 2006). Glass balls (mean weight of 0.0115 g) were carefully added on the top of the undisturbed 7 days culture until the biofilm broke or sank. A significant MDM was recorded when more than one replicate retained at least one ball for at least 5 seconds.

Statistical Analyses

Data were analyzed using one-way analysis of variance (ANOVA) to determine significant differences between means \pm standard deviation using MINITAB v18 (Minitab Ltd, UK). Significance differences were all at $p < 0.05$ even though this is not repeated in the explanations of results. Phenotypic factors (biofilm growth, attachment and strength) data was analyzed by Hierarchical Cluster Analysis (HCA) using the Ward JMP v7.0 (SAS Institute Inc., USA) method without any weighting of factors (i.e. each factor was treated equally).

RESULTS

Isolates of varying phenotypic cultural characteristics were obtained from the cowpea nodules. They varied in size, colour, and texture. The isolates were all gram-negative rods and utilized a wide range of nutrients and tolerated 10 °C and 50 °C temperature ranges (Rabiu, 2017). Table 1 shows isolates selected to form each location. Adamawa 1 had the most diverse colonies while Kano 5 had uniform colonies isolated from the nodules.

Table 1: Nodule Collection Sites and Corresponding Isolates

Location of Nodule Recovery	Isolate's Designation
Kano 1(11.67° N; 8.26° E)	1A, 2A, 3A and 4A
Bauchi(10.30° N; 9.84° E)	5B and 6B
Gombe(09.96° N; 11.16° E)	7C and 8C
Kano 2 (11.98° N; 8.35° E)	9D and 10D
Kano 3 (12.19° N; 8.63° E)	11E, 12E, and 13E
Kano 4 (11.98° N; 8.45° E)	14F, 15F, 16F, 17F and 18F
Adamawa 1(09.33°; 12.23° E)	19G, 20G, 21G, 22G, 23G, 24G and 25G
Kano 5 (12.09° N; 8.50° E)	26H
Kano 6 (11.98° N; 8.43° E)	27I and 28I
Kano 7 (11.79° N; 8.02° E)	29J and 30J
Adamawa 2 (09.20° N; 12.48° E)	31K, 32K, 33K, 34K and 35K
Kano 8 (12.11° N; 8.75°E)	36L, 37L, 38L,39L and 40L

The 40 cowpea nodular isolates qualitatively indicated varying levels of good growth (turbidity in the cultures), biofilm formation attachment (purple rings on glass vials) and strength (retention of glass beads) as shown in Plate I. Values for quantitative assessments of growth, biofilm attachment and strength are shown in Table 2. The mean growth (OD_{600}) of the isolates ranged between 1.493 (isolate 32K) and 2.101 (isolate 19G). Some significant differences in growth were observed, for example, isolate 19G produced one of the cloudiest cultures and had significantly higher growth than isolate 22G (clearer culture). However, most of the values for growth among the isolates were not significantly different. Biofilm formation is indicated by the attachment of culture to a

surface, all the isolates formed biofilms in the static microcosms. There were more significant differences in mean biofilm attachment values among the isolates than observed in growth. Isolate 30J which left the most prominent ring on the vials yielded the highest mean attachment (A_{570}) of 1.543 while vials containing isolate 9D left faint rings had a mean attachment (A_{570}) of 0.084. Isolate 30J had significantly higher biofilm attachment than all the other isolates. The isolates in this work generally formed weak biofilms according to the method used. Isolate 2A retained the highest number of glass beads and had significantly higher mean biofilm strength (0.412g) than the rest of the isolates. Twelve isolates did not form biofilms that were strong enough to hold any beads for 5 seconds.

Table 2: Mean Growth (OD₆₀₀), Attachment (A₅₇₀) and Strength (g) ± SD of Cowpea Nodular Isolates

Isolate	Growth (OD ₆₀₀)	Attachment (A ₅₇₀)	Strength(g)
1A	1.832±0.040ghijklmn	0.178±0.023lm	0.018±0.008bc
2A	1.732±0.028lmn	0.086±0.028m	0.412±0.173a
3A	1.937±0.020abcdefgghij	0.160±0.018m	0.000±0.000c
4A	1.931±0.029bcdefghijk	0.261±0.042jklm	0.000±0.000c
5B	1.929±0.032ijk	0.175±0.031lm	0.035±0.029bc
6B	1.811±0.083ijklmn	0.203±0.032klm	0.000±0.000c
7C	2.061±0.026abc	0.199±0.048klm	0.000±0.000c
8C	1.547±0.402opq	0.089±0.041m	0.002±0.008c
9D	2.002±0.013abcdef	0.084±0.010m	0.000±0.000c
10D	1.662±0.073nop	0.214±0.108klm	0.002±0.005c
11E	1.985±0.025abcdefgh	0.348±0.115ijklm	0.005±0.006c
12E	2.041±0.040abcde	0.247±0.074jklm	0.001±0.004c
13E	1.691±0.046mno	0.267±0.074jklm	0.000±0.000c
14F	1.950±0.024abcdefghij	0.293±0.061jklm	0.000±0.000c
15F	1.937±0.040abcdefghij	0.243±0.058jklm	0.000±0.000c
16F	1.991±0.043abcdefg	0.663±0.183efgh	0.017±0.006bc
17F	2.045±0.050abcd	0.240±0.047jklm	0.012±0.007c
18F	1.973±0.051abcdefghi	0.164±0.035lm	0.028±0.022bc
19G	2.101±0.011a	0.237±0.048ijklm	0.017±0.022bc
20G	2.086±0.031ab	0.226±0.050klm	0.012±0.007c
21G	1.912±0.037cdefghijk	0.332±0.165ijklm	0.007±0.005c
22G	1.763±0.106klmn	0.277±0.086ijklm	0.008±0.008c
23G	1.786±0.079ijklmn	0.296±0.089ijklm	0.010±0.009c
24G	2.007±0.017abcdef	0.665±0.280defgh	0.000±0.000c
25G	2.010±0.066abcdef	0.568±0.136efghi	0.002±0.008c
26H	1.982±0.014abcdefgh	0.568±0.136efghi	0.000±0.000c
27I	1.875±0.061efghijkl	0.499±0.220ghij	0.001±0.004c
28I	2.016±0.019abcdef	1.171±0.176b	0.000±0.000c
29J	1.866±0.128fghijkl	0.758±0.312defg	0.002±0.005c
30J	1.832±0.136ghijklm	1.542±0.213a	0.000±0.000c
31K	1.730±0.1275lmn	1.079±0.357bc	0.015±0.008bc
32K	1.493±0.139q	0.856±0.180cde	0.008±0.011c
33K	1.886±0.032defghijkl	0.432±0.074hijkl	0.017±0.008bc
34K	1.889±0.039defghijkl	0.739±0.111defg	0.011±0.000c
35K	1.820±0.050hijklmn	0.851±0.126cde	0.027±0.012bc
36L	1.509±0.034pg	0.458±0.087hijk	0.010±0.004c
37L	1.915±0.023cdefghijk	0.866±0.101cde	0.073±0.043b
38L	2.087±0.022ab	0.9345±0.1642bcd	0.008±0.008c
39L	2.013±0.044abcdef	0.7850±0.1537def	0.015±0.008bc
40L	1.897±0.026cdefghijkl	0.763±0.145defg	0.037±0.021bc

Variations in growth and biofilm characteristics of the isolates analyzed using HCA to group the isolates are shown in the constellation (Fig. 1). The shorter the distance between the isolates, the more similar they are.

Group I(11E, 16F, 25G,38L,39L, 24G, 26G, 28I) had higher mean values for the three biofilm parameters. Group II(40L, 37L,36L,35K,34K,33K,32K,31K,29J, 27I,21G) performed better in growth and

attachment. Isolates in group III (12E,17F,18F,19G,20G,5B,14F,15F,3A,4A,7 C,9D) had low mean values in attachment and strength while 9 isolates in group IV had very low to zero mean values in attachment and strength.

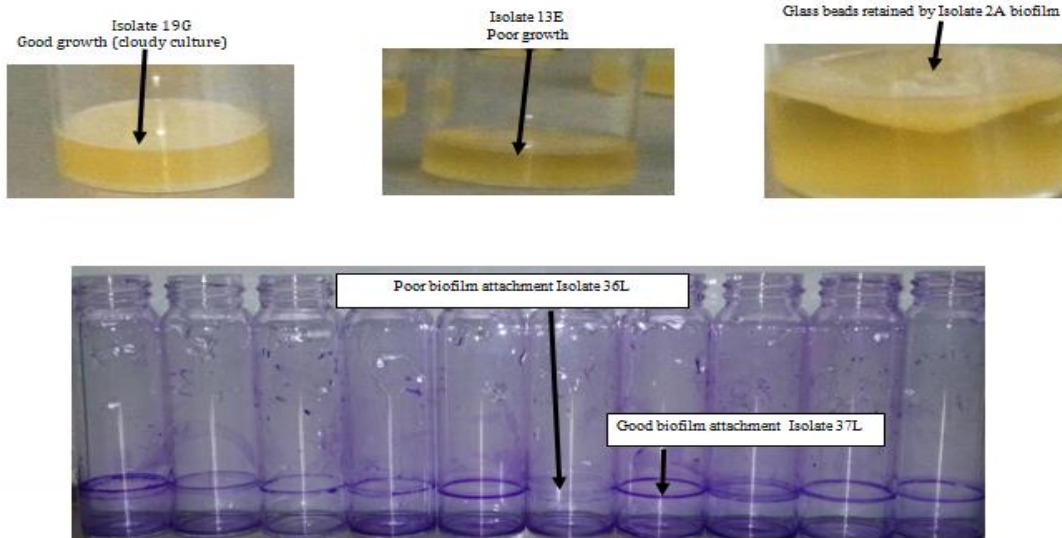
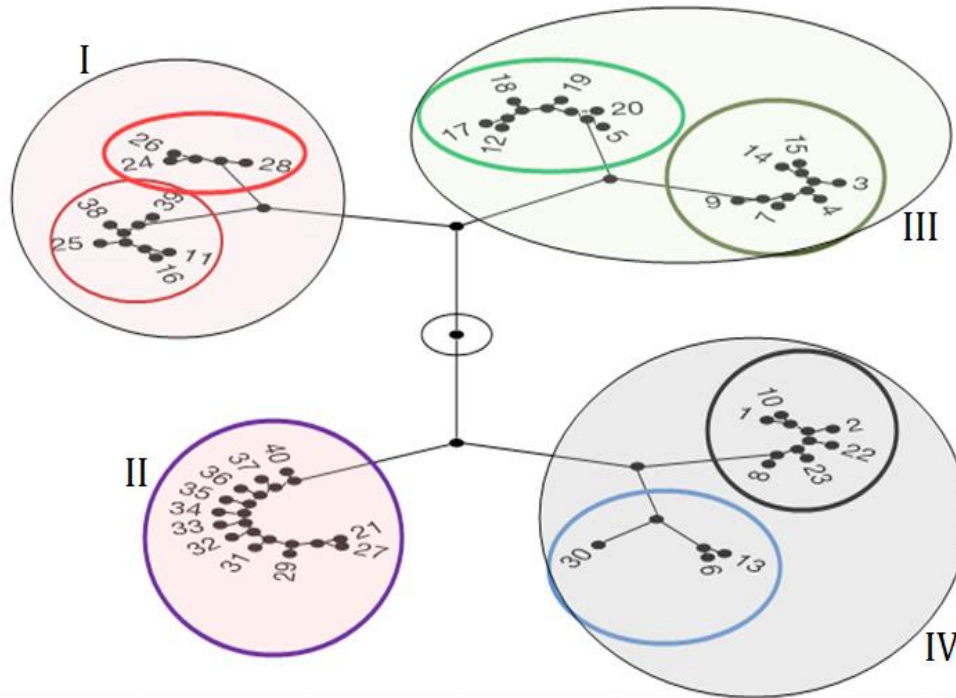


Plate I: Differences in Biofilm Characteristics of Cowpea Nodular Isolates: 19G (good growth), 13E (poor growth), 2A(strength, 36L(Poor attachment and 37L(good attachment)



Key: 1-4 A, 5-6 B, 7-8 C, 9-10 D, 11-13 E, 14-18 F, 19-25 G, 26 H, 27-28 I, 29-30 J, 31-35 K, 36-40 L
I-highest mean values for biofilm parameters, **II**-High mean values for growth and attachment, **III**- Low mean values in attachment and strength, **IV**-Very low to zero mean values in attachment and strength
Fig 1: HCA Analyses Constellation Plot of Biofilm Characteristics of Cowpea Nodular Isolates

DISCUSSION

Isolates used in this research are symbionts of cowpea which makes them endophytes of interest in screening and characterization for plant growth promotion. Biofilms can accumulate metals, associated with the emergence of antibiotic resistance and promote the evolution and genetic diversity of natural microbial communities (Chadha, 2014). Therefore, biofilm formation has biotechnological significance. All 40 isolates were able to grow and attach to static microcosms. The formation of biofilm by root-associated bacteria has also been reported by Altaf and Ahmad (2016) and Gao *et al.* (2015). Li *et al.* (2017) showed that 100% of isolates from the roots of a desert plant *Lepidium perfoliatum* form a biofilm no matter the stress conditions which may assist them in colonization.

The isolates showed varying rates of growth indicated by differences in turbidity which corresponded to the OD600 values. The degree of turbidity in the broth culture is directly related to the number of microorganisms, studies have shown that cell density is proportional to OD600 according to Domańska *et al.* (2019). Udall *et al.* (2015) reported lower values for growth (0.77– 1.80 OD600) by environmental *Pseudomonas* after 3 days of incubation. The lower values could be due to the differences in incubation periods and the sources of the isolates. The cowpea nodular isolates in this research showed greater attachment than reported by Li *et al.* (2017); 0.171 to 0.749 A570 and Udall *et al.* (2015); 0.17–0.27 A570. Udall *et al.* (2015) reported attachment (0.17–0.27 A570). Endophytes are soil microorganisms that successfully colonize plants; these results demonstrated that biofilm formation has significance in colonization. Biofilms are probably among the strongest deterministic factors that determine the success of bacteria

to become endophytic (Czaban *et al.*, 2007; Hardoim *et al.*, 2008; Kearns, 2010). Biofilm formation is indicated in host specificity, participation in the early stages of a host plant infection, signalling molecule during plant development, and protection from environmental stresses (Nocelli, 2016). The isolates did not form biofilms that were assessed as strong. Li *et al.* (2017) showed that one strain, belonging to the Bacillaceae family, presented a strong biofilm compared to pseudomonads in their studies. In contrast to this research, Udall *et al.*, (2015) recorded greater values for strength (0.6–0.75) using the same method as in this work. Likely, endophytes are not challenged by environmental perturbations necessitating the production of stronger biofilms for protection. The variations in growth, biofilm attachment and strength observed could be due to the type of biofilm formed (Spiers *et al.*, 2006). The diversity of biofilm characteristics indicated that cowpea nodules contain other bacterial species in addition to well-known rhizobia (Martinez-Hidalgo and Hirsch, 2017). Genotypic variations among the isolates may have led to their differences in biofilm characteristics, Rinaudi and Giordano (2010) and Ramey *et al.* (2004) reported that nodular bacteria (rhizobia, pseudomonads) as well as other plant-associated bacterial species form varying biofilm structures on plant surfaces. Rossetto *et al.* (2011) also observed variation in biofilm formation in two endophytic strains of *Methylobacterium* spp. on sugarcane roots and inert wooden sticks. Differing biochemical test results suggest some underlying differences in the genomics of the isolates (Rabiu, 2017).

CONCLUSIONS

This work supports that biofilm formation is linked to phenotypic behaviours of plant-associated bacteria and is therefore a tool for characterization before selection for plant

growth promotion. All the isolates showed good growth in static microcosms, varying levels of attachment and mostly weak biofilms. Isolates from all 12 locations possess at least one good biofilm parameter.

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