Advances in Biological Research 2 (5-6): 90-96, 2008
ISSN 1992-0067

Physiological Studies on *Xanthomonas axonopodis* Pv *Manihotis* (*Xam)* Strains Isolated in Nigeria

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Abstract: *Xanthomonas axonopodis* pv manihotis (*Xam*) is the causal agent of cassava bacterial blight (CBB) worldwide. To develop a well-organized disease management policy in a country, the diversity of pathogens population must be known. The bacterial populations of *Xanthomonas axonopodis* pv *manihotis* (*Xam*) obtained as flora cassava leaves and stems, in Nigeria were characterized by their biochemical and the physiological reactions with basic routine microbiological techniques which were subjected to cluster analysis. A total of 75 bacteria strains were studied, made up of sixty eight (68) *Xanthomonas axonopodis* pv manihotis (*Xam*), four (4) *Xanthomonas axonopodis* pv cassavae (*Xac*) and three (3) unidentified species. The isolates were similar to one another in most of the cultural and physiological characteristics. They were all able to hydrolyze aesculin and showed a positive reaction to catalase, citrate and oxidase tests. They were indole, methyl-red, Voges-Proskauer and urease negative. The analysis carried out on the biochemical data clustered the *Xam* and *Xac* together at 94.5 %; the unidentified strains were separated. The dendrogram generated from the data obtained from the oxidative metabolism of various sugars clustered the pathogens together at 94 %, also exempting the unidentified bacterial flora. We conclude that cultural and physiological reactions could not differentiate the yellow strains from the white strains of the pathogen of cassava bacterial blight from the result obtained in this study. Although there are variations in the reaction of the pathogen to some tests conducted, these could not be used to segregate the bacteria into different pathogenic group since they are not consistent within the white strains of the bacteria.

Key words: Pathogen · Cultural and Physiological Characteristics · Oxidative Metabolism · Dendrogram

INTRODUCTION

The causal agent of cassava bacterial blight is *Xanthomonas campestris* pv *manihotis* (synonym *Xanthomonas axonopodis* pv *manihotis* [1,2]. The pathogen was first reported in Brazil and later observed in several countries of Southern America, Africa and Asia [3, 4]. Two pathovars of *Xanthomonas axonopodis* are pathogenic to cassava and cause different diseases [5]. *Xanthomonas axonopodis* pv *manihotis* is the causal agent of cassava bacterial blight while *X. axonopodis* pv *cassavae* is associated with bacterial necrosis of cassava. Maraite [5] described the symptoms of *X. axonopodis* pv *manihotis* infection which include angular leaf spots, blight, production of exudates, wilting, vascular necrosis of the stem and die-back of the stem. The combination of diverse symptoms by the pathogen of cassava bacterial blight is unique among diseases caused by plant pathogenic bacteria.

Lozano [6] reported that Bondar [7] was the first to report the incidence of cassava bacterial blight in Brazil as a bacterial disease of cassava. The causal organism of cassava bacterial blight disease was first named *Bacillus manihotis* (Arthaud-Berthet) Starr [6] later *Phytomonas manihotis* (Arthaud-Berthet and Bondar) Viegas. The name was changed to *Xanthomonas manihotis* (Arthaud-Berthet) Starr and one edition of Bergey's Manual listed over a hundred different pathogens including *Xanthomonas cassavae* (yellow colony bacterium) and *Xanthomonas manihotis* as pathovars under *Xanthomonas campestris* [8]. Most recent nomenclature categorized *X. campestris* pv *manihotis* as a synonym of *X. axonopodis* pv *manihotis* (*Xam*) [2].

Xanthomonas axonopodis pv *manihotis* (*Xam*) is a Gram negative rod which does not form spores or capsules. The bacterium grows on sucrose–containing media producing non-pigmented colonies, usually motile with a single polar flagellum. Except for the

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absence of pigmentation, most physiological and biochemical characteristics of this bacterium are those of Xanthomonads [9, 3]. The bacterium is catalase positive, hydrolyzes milk and aesculin aerobically and utilizes sodium polypectate but it is negative to indole, methyl red and the Voges-Proskauer test. It is also phenylalanine, deaminase and urease negative. Asparagine is not utilized [10].

Ikotun [9] tested the ability of the two bacteria pathovars (*Xanthomonas campestris* pv *manihotis* and *Xanthomonas campestris* pv *cassavae*) to utilize various carbon and nitrogen compounds as their sole source of carbon and nitrogen. He found that the bacterial isolates utilized a range of sugars, organic acids, amino acids, amines and some inorganic nitrogen sources for growth which slightly differentiated *Xanthomonas axonopodis* pv *manihotis* from *X axonopodis* pv *cassavae.*

This study thus focuses on the biochemical characteristics of this bacterial population in Nigeria to ascertain the reliability of their physiological properties.

MATERIALS AND METHODS

Isolates Collection: Field isolates of bacteria collected in Nigeria in 2000 [11] were used in this study. The bacterial were isolated and identified as described in previous work [12,13]. The States and locations of collection of diseased cassava plants from which the bacterial strain were obtained are shown in Fig. 1. The bacteria were cultured on yeast extract dextrose peptone agar (YDPA) containing 5g yeast extract, 10g dextrose, 5g peptone and 15g agar/L of distilled water (pH 7.2). The medium used for short-term storage of the bacteria culture was Yeast Glucose Calcium Agar (YGCA). All cultures of the bacterial isolates were freeze-stored in 60% glycerol inside 1.5 ml Eppendorf tubes and were kept in a -80°C freezer for long-term storage.

Morphology of Bacterial Isolates: The bacterial cells were cultured overnight on nutrient agar plates. Each bacterial isolate was smeared with a drop of water on a cleaned, grease-free glass slide and air-dried. The prepared slides were Gram stained as suggested by Olutiola *et al.* [14]. The stained slides were examined under a light microscope with oil immersion objectives and the picture was captured. The hanging-drop method described by Olutiola *et al.* [14] was used to determine motility in the bacterial isolates.

Biochemical and Physiological Tests: Basic routine microbiological techniques were employed to determine the ability of the bacterial isolates to utilize, hydrolyze and liquefy various chemical substances. Catalase reaction and indole test were done according to the method described by Olutiola *et al.* [14] and Ogunjobi [12]. Oxidate test was carried out as described by Kovacs [15] and the method of Fahy and Hayward [16] was use to detect casein utilization, starch hydrolysis and the presence of enzyme urease. The methods cited by Skerman [17] were used for methyl red, Voges Proskauer, nitrogen reduction and citrate utilization tests. Hydrogen sulphide production and gelatin hydrolysis were carried out using a modified method of Hayward and Hodgkins [18]. The method of Sneath [19] was employed to detect aesculin hydrolysis ability of the isolates. The modified method of Thornley [20] was used to detect the anaerobic breakdown of arginine. Acid production from carbohydrates was determined using a modified method of Fahy and Hayward [16].

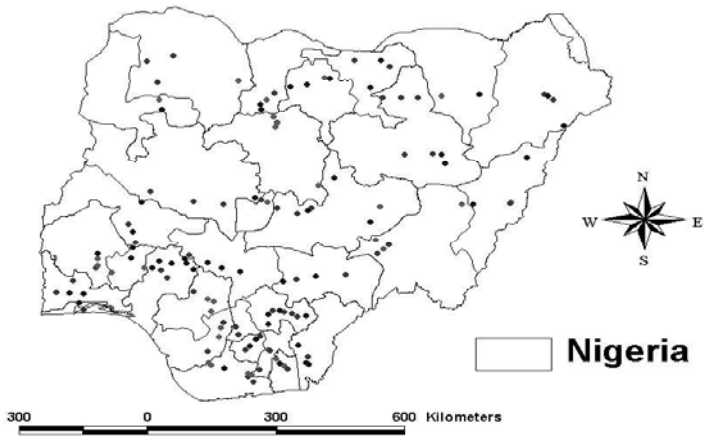


Fig. 1: The origin of diseased cassava leaves and stems from which bacterial strains were obtain in Nigeria

The growth patterns of the bacterial isolates were observed on different media to study their characteristic nature of the bacteria on plate culture.

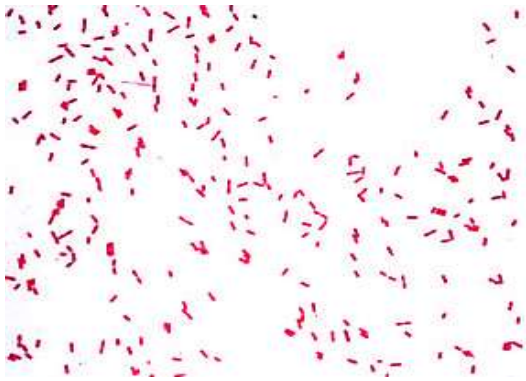
Data Analysis: The results obtained in this study were scored as zero for negative reactions and one for positive reactions based on the presence or absence of a particular physiological character in the bacterial strains and analyzed using Un-weighted Pair Group method of Arithmetic Average Clustering (UPGMA) of Numerical Taxonomy and Multivariate Analysis System (NTSYS-PC 2.0 version) computer software to generate a phylogenetic tree from the data.

RESULTS

The bacterial isolates causing bacterial blight of cassava were very similar to one another in most of the cultural and physiological characteristics. The results of cultural and physiological reactions obtained in this study could not differentiate the yellow strains from the white strains of cassava bacterial blight pathogen. Morphological and biochemical characterization of bacterial isolates revealed that the cassava bacterial blight causal agent is a Gram-negative rod. The photomicrograph of the bacterium is shown in Plate 1.

The bacteria were motile and aerobic, non-fluorescent in King's medium B, but grew with characteristic mucoidal colonies on media containing sucrose. Most of the bacterial isolates liquefied gelatin and few were unable to utilize gelatin. Also, a variable reaction was observed with arginine hydrolysis and casein utilization among the *Xam* isolates. They were able to hydrolyze aesculin and were catalase, citrate and oxidase positive. They were indole, methyl-red, Voges-Proskauer and urease negative. Hydrogen sulphide production from cysteine and nitrate reduction to nitrite showed variable reactions. Starch was also not utilized by these pathogens. These bacteria oxidatively metabolized glucose, mannose, arabinose, trehalose, cellobiose and fructose. They were also able to produce acid from maltose, xylose and galactose. Variable reactions were, however, seen in acid production from some carbohydrates, which included lactose, sucrose, rhamnose and raffinose. They could not oxidatively metabolize inulin, mannitol and sorbitol.

All the *Xanthomonas axonopodis* pv *manihotis* strains produced greyish-white, smooth, glistening mucilaginous convex colonies on nutrient agar, nutrient sucrose agar, sucrose peptone agar and glucose-yeast extract-calcium carbonate agar. They were not mucoidal on nutrient agar but produced mucoidal colonies on sucrose peptone agar and did not produce any pigment



Magnification: X 100

Plate 1: Microscopic view of Gram stained *Xanthomonas axonopodis* pv *manihotis*

on all the above media. Slight pinkish pigmentation occasionally appeared when bacteria were stored on glucose-yeast extract-calcium carbonate agar after 6 weeks in the cool room at 4°C. The pigmentation, however, was not consistent with the same set of strains in subsequent experiments. The yellow variants of the bacteria formed colonies similar in appearance to those of *Xam,* apart from their pale yellow pigmentation. The yellow variants also varied in their rate of growth. After 48 hours of incubation on nutrient agar, the *Xam* colonies were 1.5-3.0 mm in diameter, while those of the yellow strains were 0.5-2.0 mm. It thus seems that the non-pigmented grew faster than the yellow variants.

The analysis of the biochemical data obtained showed a very close relationship among the bacterial populations. At 94.5% similarity coefficient, all the *Xanthomonas axonopodis* pv *manihotis* and *Xanthomonas axonopodis* pv *cassavae* were clustered together, except strain 98 which came into the cluster with the others at 88% coefficient of similarity. The three unidentified bacteria strains, designated 74A, 105B and 101A, were the only ones that had a distant relationship with the *Xam* strains. However, at a higher similarity coefficient of 95% and above, nine clusters were alienated from the dendrogram and four strains were not clustered. These were strains designated 80, 131A, 98 and the unidentified 101A as shown in Fig. 2. Cluster 3 had the highest population of 30.6% of *Xam* strains clustered together with 100% similarity; 28% of the bacterial strains were assembled in cluster 2 showing the same 100% similarity in biochemical and physiological reactions. Most of the strains in the other clusters were different from the strains in the major cluster at one or two physiological reactions. For instance, bacterial strains in cluster 1 were able to utilize casein among the *Xam*

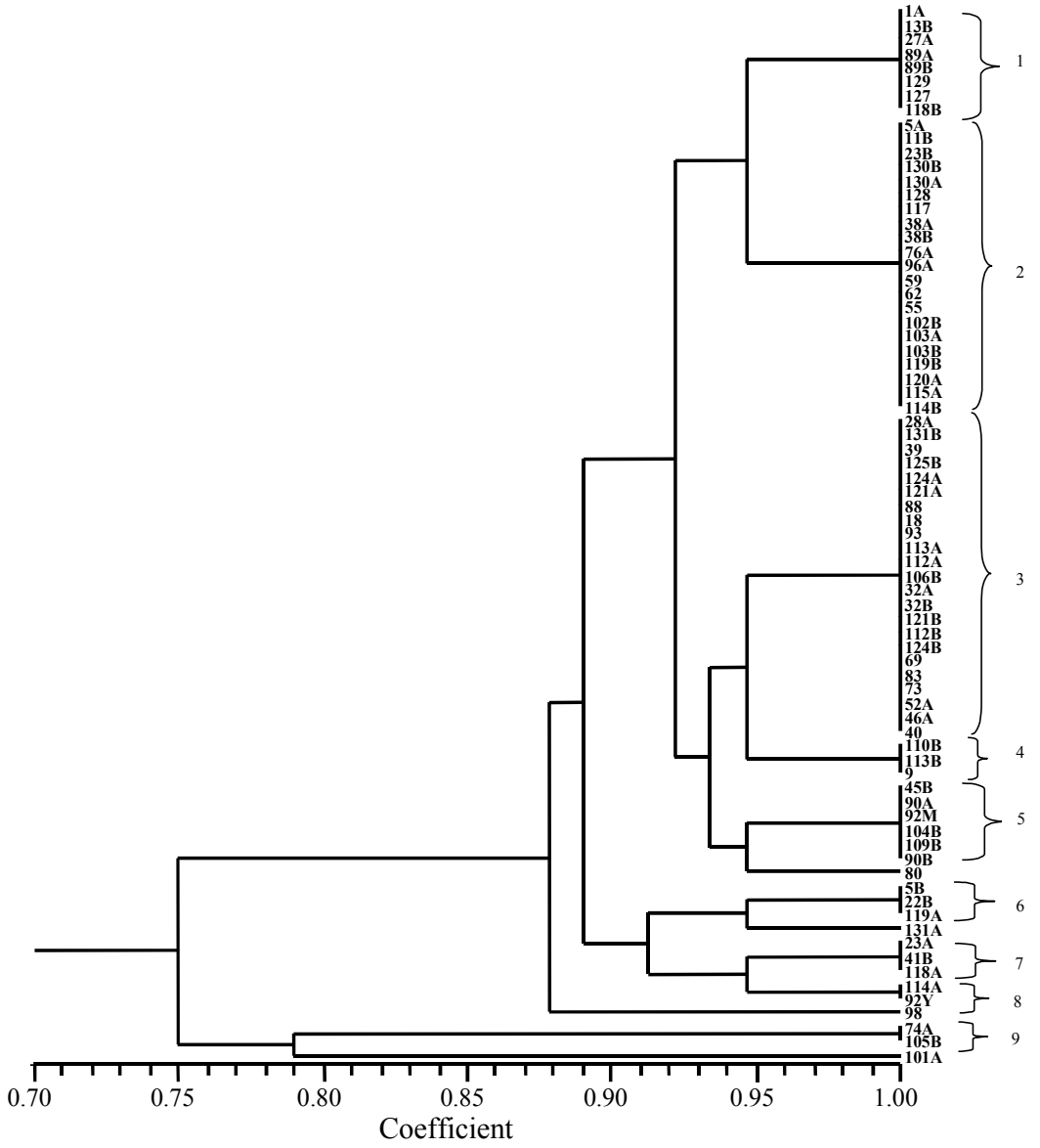


Fig. 2: Dendogram of biochemical characteristics of *Xanthomonas axonopodis* pv *manihotis* population in Nigeria

isolates while those in cluster 2 did not utilize casein. Those in cluster 3 were different from others in their inability to hydrolyze arginine. All the three strains in cluster 4 were unable to liquefy gelatin.

The dendrogram generated from the oxidative metabolism of various sugars showed that the bacterial populations are very closely related (Fig. 3). At 94% coefficient level of similarity all the *Xam* and *Xac* strains were clustered together except two strains of *Xam* (39 and 92M) and the three unidentified bacteria species. At a higher coefficient level of similarity, nine clusters were identified and five strains were separated from the others. Cluster 2 grouped the 28% of the *Xam* strains together, forming the largest cluster. This was followed by cluster 6 with 18.6% of the bacterial population. The difference observed in the clusters of the bacterial populations has to do with ability of some strains to metabolize one or two carbohydrates than others strains. All the bacterial in cluster 1 were unable to produce acid from rhamnose and sucrose while cluster 2 grouped all the strains that could not metabolize rhamnose but produced acid from sucrose metabolism. Biochemical and physiological reactions cannot differentiate the two pathovars of this genus of bacterium. The four yellow variants designated 90A, 90B, 92Y and 93 were separated into different clusters in the

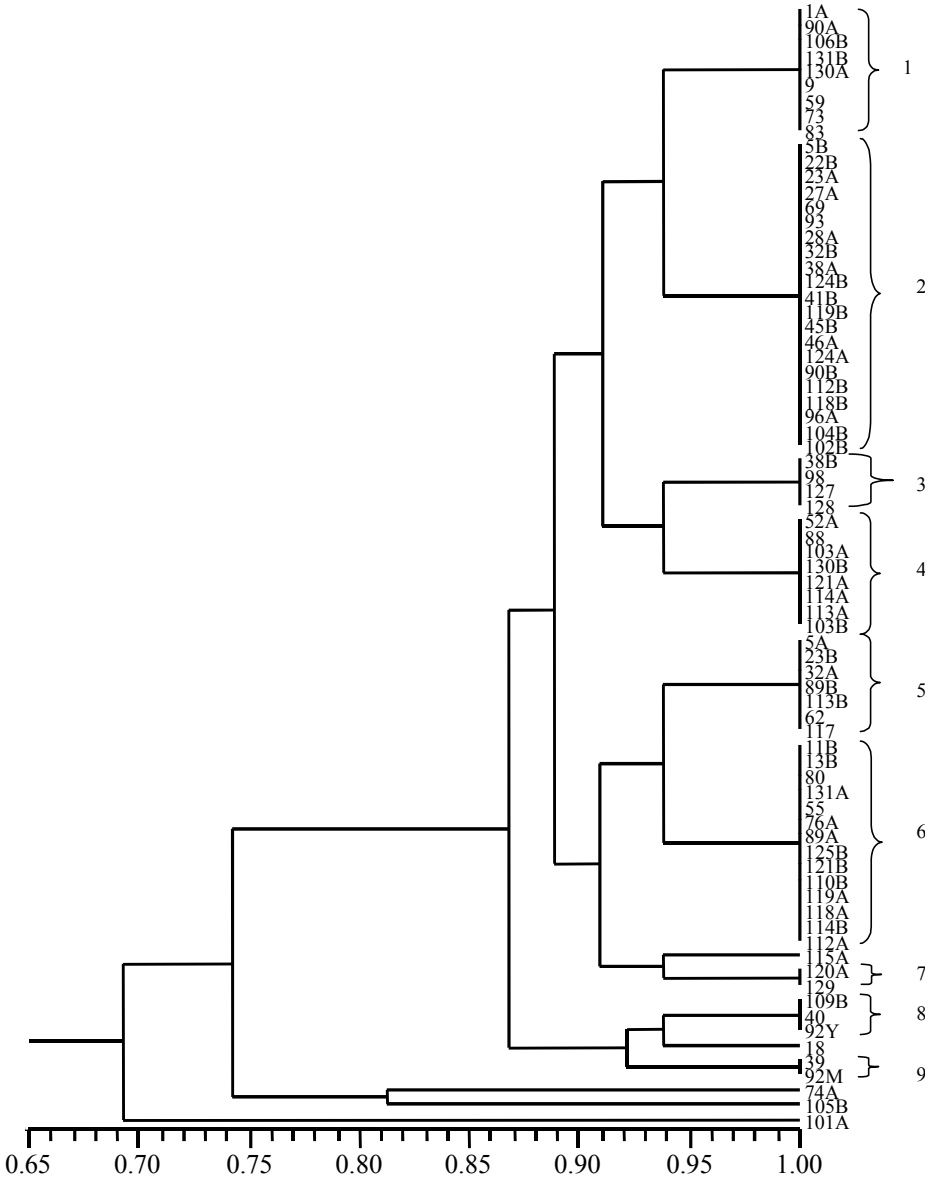


Fig. 3: Dendrogram of utilization of carbohydrates by *Xanthomonas axonopodis* pv *manihotis* population in Nigeria

first dendrogram. Strains 90A and 90B were grouped in cluster 5 while 92Y was in cluster 8 and 93 in cluster 3. In the second assemblage, (Fig 3) the *Xac* strain 90A was grouped in cluster 1, 90B and 93 were grouped in cluster 2, while 92Y was in cluster 8.

DISCUSSION

The bacterial isolates causing bacterial blight of cassava were very similar to one another in most of the cultural and physiological characteristics. The results of cultural and physiological reactions obtained in this study could not differentiate the yellow strains from the non-pigmented strains of the bacteria causing cassava bacterial blight. There are conflicting reports on the physiological reactions of these two bacterial pathovars. Ikotun [21] reported that there were differences in the starch hydrolysis among the isolates of *Xanthomonas axonopodis* pv *manihotis* isolated in Africa compared to the South American strains, as African strains were said to be weak starch hydrolyser while South American strains attacked starch readily. The ability of the strains obtained in this study to hydrolyze starch could not be established. This was contrary to the early report of Ikotun [21] on the inherent ability of African strains to utilize starch.

In this study, all the non-pigmented strains and the yellow variants were able to grow and produce acid from maltose. This agreed with the findings of Ikotun [9]. The yellow variants were not able to produce acid faster from maltose than the other strains which was contrary to the findings of Ikotun [9]. Hydrogen sulphide production was not consistent in all the isolates but 87.5% (63 strains) were positive in being able to produce hydrogen sulphide from cysteine peptone broth. This agreed with the report of Persley [10] who found similar results in her work on the biochemical reactions of the bacteria. Other physiological tests done by Maraite and Weyns [22] and Ikotun [9] to differentiate the two strains were either unstable (for example, nitrite production from nitrate and acid production from lactose) or inconsistent amongst the non-pigmented *Xanthomonas axonopodis* pv *manihotis* (acid production from raffinose, sucrose and rhamnose). Considerable variation has also been observed among *Xam* isolates in relation to biochemical and physiological characteristics [23].

The variations observed in the physiological reactions were not enough to differentiate these bacterial populations into strains of different characters. They also did not have any correlation with the pathogenic behavior of the pathogens [13]. To better understand the pathogenic behavior of the bacterial population structure and to know the diversity within the population of the bacteria as a result of host selection pressure, environmental factors and genetic alteration, molecular techniques that rely on the amplification of specific primers on the DNA of the bacterial strains are suggested. Molecular fingerprinting has be proved to differentiate the yellow variants from the non-pigmented Cassava Bacterial blight casual agent *Xanthomonas axonopodis* [12, 24].

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