Occurrence and Pathogenic Variation of *Xanthomonas axonopodis* pv. *vignicola* in Selected Locations in Northern Nigeria

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Abstract

Efforts to reduce losses in cowpea through breeding and screening for resistance to Xanthomonas axonopodis pv. vignicola (Xav) have not been successful due to occurrence of pathogenic variation among the pathogen in different locations. In order to study the existence of pathogenic variation in Xanthomonas axonopodis pv. vignicola in cowpea producing regions in Nigeria, seed samples were collected from nine cowpea producing states and three cowpea seed companies. The disease incidence was determined by plating four hundred seeds from each seed lot in Yeast Dextrose Carbonate Agar (YDCA). Colonies with typical characteristics of Xav were selected for pathogenicity testing on Ife brown, Sampea7 and IT86D-721 cowpea varieties. The isolates were further tested for morphological and biochemical reactions. The experiment was laid out in Complete Randomized Design with five replications and repeated in a second trial. Seeds on which there was Xav growth were recorded as positive and those on which there was no Xav growth were recorded as negative. Xav was isolated from seed lots collected from nine cowpea producing states and three cowpea companies. Colonies that hydrolyze starch on YDCA were assumed to be Xav. Pathogenicity test revealed significant variation among the isolates both in disease incidence and severity and time taken (3-7 days) to produce the typical blight symptoms. All the isolates demonstrated variable level of virulence on all the tested cowpea cultivars. The high incidence rate and virulence status of the isolates show that cowpea bacterial blight is indeed a threat to the production of cowpea in all the regions.

Keywords: Bacterial blight, Isolation, Occurrence, Pathogenic Variation, Virulence.

Introduction

Cowpea bacterial blight (CoBB) remains one of the most damaging diseases of cowpea (Okechukwu *et al*., 2010). In the tropics and subtropics, it frequently and severely attacks the crops and it is seed-transmitted (Singh and Munoz, 1999). Four bacterial diseases have been documented to attack cowpea, two of them are of economic importance and they are bacterial blight and postule induced by *Xanthomonas axonopodis* pv. *vignicola* (Vauterin, 1995; Emechebe and Florini, 1997; Shoaga, 1998). Bacterial blight is the most wide spread disease of cowpea, which has been reported from all regions of the world in which cowpea is cultivated (Emechebe and Florini, 1997). The pathogen attacks all aerial plant parts including leaves, petioles, pods, stems and seeds, but the characteristics symptoms of chlorotic border around the necrotic lesions are more severe and conspicuous on leaves of all susceptible cultivars (Goodwin *et al*., 1995). In areas where cowpea is commercially grown, yield depression due to CoBB may be as high as 71 % in pod, 68 % in seeds and 53 % in fodder (Okechukwu *et al.*, 2000).

Various chemical and cultural means of diseases management, including the production of clean seed, have been developed but none is likely to be fully practicable in developing countries in the tropics where the disease is severe (Saettler, 1989; Opio *et al*., 1993). Strains within the *Xav* species complex are so diverse, hence, the development of a universal control method difficult. Attention is being paid to Cowpea production increase, and this may be a direct benefit of finding resistant varieties to the disease. Genetic and pathogenic variations in phytopathogenic bacteria have been cited as obstacles to the development of resistant varieties (Yu *et al.,* 1998). Genetic variation among *Xanthomonas axonopodis* pv. *vignocola* have been reported (Verdier *et al*., 1998, Mhedbi-Hajri *et al*., 2013). Bactericides and many fungicides are reported to inhibit bacterial diseases but their bio-efficacy and suitability varies with location and variation among the pathogenic bacteria. There is need to verify the existence of pathogenic variation in *Xav* both in *in vitro* and field studies, in order to incorporate the knowledge in the management package of the disease (Nandini, 2012). The current information on the occurrence and the pathogenic variation among the different isolates of the pathogens in cowpea producing regions in Nigeria is generally lacking. The present study was designed to investigate the occurrence and pathogenic variation in *Xav*, the causal organism of CoBB in cowpea in selected locations of Northern Nigeria.

Materials and Methods

Seeds were obtained from the open markets in nine cowpea producing states (three states each from North-east, North-central and North-west). The sites where the seed lots were collected were Bama market in Borno, Akor market in Gombe, Mubi market in Adamawa, Duchin Kura market in Sokoto, Jega market in Kebbi, Saminaka market in Kaduna, Keffi market in Nasarawa, Mokwa market in Niger and Idah market in Kogi state. The seed companies were Alheri, Masalaha, and Premier which distribute cowpea seeds to these Northern states. The criteria for seed selection were based on the absence of visible damage and on cleanliness; the selected seeds were clean, with no obvious symptoms of disease.

Incidence of *Xanthomonas axonopodis* pv. *vignicola* in seed samples

The incidence of *Xav* in seed samples was determined by plating 400 seeds from each of the seed lots in YDCA (Yeast Dextrose Calcium carbonate Agar). YDCA contains Yeast extract - 10.00 g, D-glucose - 20.00 g, $CaCO_3$ - 20.00 g, Agar - 15.00 g, Distilled water - 1000 ml.

All ingredients were mixed except $CaCO_3$ and sterilized in an autoclave. $CaCO_3$ was later added to the media and thoroughly mixed. Twenty ml of the agar was poured into each Petri-dish, then allowed to cool and solidify. Twenty cowpea seeds were plated per Petri-dish. The experiment was laid out in a Complete Randomized Design (CRD) with twenty replications. The plates were incubated at 28°C for 72 h and observed for the production of typical colonies which should be mucoid, round, convex, glistening, slimy and yellow. The suspected colonies were isolated in pure culture and a four-fold serial dilution was made on YDCA (Schaad and Stall, 1988). The procedure was repeated in the second trial. Incidence was taken by observing typical *Xav* yellow colony on the plates and the population was calculated by counting the number of typical *Xav* colony on the plates as shown in formula below:

$$\text{Incidence} = \frac{\textit{Numbers of plates showing typical yellow colony}}{\textit{Total number of plates inoculated}} * 100$$

$$\text{Population} = \frac{\textit{Numbers of CFU}}{\textit{Volume plated (ml)}} * \textit{total dilution used}$$

Morphological characterization

The morphological characters observed were shape (round), Gram reaction and pigmentation characters as described by Society of American Bacteriologists, Bradbury (1970) and Schaad and Stall (1992). Potassium hydroxide solubility test (KOH) (3 % aq., w/v) was used to test the Gram reaction. A drop of KOH was placed on a sterilized slide using a pasteur pipette. A single colony was removed from agar medium using sterile loop into the KOH solution and mixed until an even suspension was obtained. Presence of mucoid, thread-like strands when the loop was lifted up confirmed the isolate as a Gram negative bacterium.

Biochemical characterization

The biochemical tests such as hydrolysis of starch, gelatin liquefaction, hydrogen sulphide production, catalase and acid production from lactose, sucrose, maltose, dextrose and mannitol by the pathogen were studied as per the methods described by Schaad (1992).

a) ***Gelatin liquefaction***

Preparation of the nutrient gelatin medium: Peptone 10.0 g, Beef extract 5.0 g, Gelatin 20.0 g, all the ingredients were mixed and volume was made to 1L with pH 7.0 and heated over a water bath until the gelatin was dissolved and sterilized for 15 min using an autoclave. The media was cooled and poured into the Petri-dishes and allowed to solidify; then, spot inoculated with 48 h culture of the test bacterium then the plates were incubated at 20 ºC. The surface of the plate was then flooded with 0.2% mercuric chloride solution of dilute HCl (20%) and observed for formation of white precipitation.

b) ***Catalase test***

A loopful of 24-48 h culture of the test bacterium was smeared on a slide and covered with a few drops of 20% hydrogen peroxide. The reaction was regarded as positive if gas bubbles are produced.

c) ***Hydrogen sulphide (H_2S) production***

The peptone water medium comprising of Peptone 10 g, NaCl 5.0 g, water 1000 ml and pH 7.0 was dispensed in 5 ml quantities in tubes and autoclaved. To detect H_2S, the lead acetate test strips were prepared as follows. Whatman No. 1 filter paper was cut into 5 × 50 mm strips which were then soaked in warm saturated solution of lead acetate. The strips were then dried, autoclaved and again dried at 60°C. The medium in each tube was inoculated with a loopful of 48 h slant growth of the test bacterium. After inoculation, the test strip was inserted in between the plug and inner wall of the tube, so that it hangs just above the broth. The tubes are incubated at 25°C and the observations were recorded at regular intervals for up to 14 days. The blackening of test strip indicates liberation of H_2S.

d) ***Starch hydrolysis***

The medium employed is referred to as starch broth and contains, Peptone (10.0 g), beef extract (5.0 g), starch soluble (2.0 g), agar (20.0

g), water (1000 ml) and pH (7.0). The starch broth was sterilized by autoclaving and poured into sterilized Petri-dishes. It was allowed to solidify and spot of the test culture was made in four plates. The plates were inoculated at 25ºC and tested for starch hydrolysis, one plate at a time, after 2, 4, 7 and 14 days. The agar surface was flooded with Lugol's iodine and allowed to act for a few minutes for development of colorless zone around the bacterial growth which indicated starch hydrolisis.

e) ***Lactose utilization***

The carbon source (lactose) was filter sterilized and mixed with autoclaved and cooled Dye's medium along with 1.2 % purified agar. The pH was adjusted to 7.2. Bacterial isolates were spot inoculated with replica plating method and incubated at 30°C for 3, 7 and 14 days. Growth was compared with control, where carbon source was not supplemented (Schaad, 1992).

f) ***Acid production from Sucrose, Maltose, Dextrose and Mannitol***

The acid production by the pathogen *Xanthomonas axonopodis* pv. *vignicola* was tested by using medium C of Dye. Ten ml of medium C of Dye was dispensed in each test tube and sterilized in an autoclave for 15 min at 121°C. Filtered sterilized carbohydrates (Sucrose, Maltose, Dextrose and Mannitol) were added to the tubes at 0.14% concentration. The tubes were inoculated with 0.1 ml of 24 h old bacteria culture and incubated at room temperature for three days. Change in the colour of the medium confirmed the acid production.

Pathogenicity tests

Seedlings of three cowpea cultivars (Ife brown, SAMPEA-7 and IT86D-721) were planted in 25 cm clay pots in a screen house at the Institute for Agricultural Research (IAR), under ambient temperature (30-31°C). Five plants per pot were planted and later thinned to three plants per pot. The experiment was laid out in a Complete Randomized Design (CRD) with five replications. The one hundred and twenty (120) *Xav* isolates for inoculation were cultured on YDCA to a log phase for 48 h. Single colonies were suspended in 0.01M phosphate buffer (pH, 7.2). The bacterial inoculum $4.5x10^7$ cfu/ml was used to inoculate the second trifoliate leaves at 14 days after sowing by spraying the bacteria suspension on the abaxial and adexial surfaces of each leaf which was allowed to run off using hand held atomizer. Afterwards, the plants were covered with transparent polythene bags for two days. The data collected were incidence, severity and time taken for typical symptom development. Disease incidence was taken by counting the number of infected plants and severity was scored using modified CIAT 1-9 scale (Opio *et al*., 1993) as shown in Figure 1. The procedure was repeated in the second trial.

$$\text{Incidence} = \frac{Numbers\ of\ infected\ plants}{Total\ number\ of\ plants} * 100$$

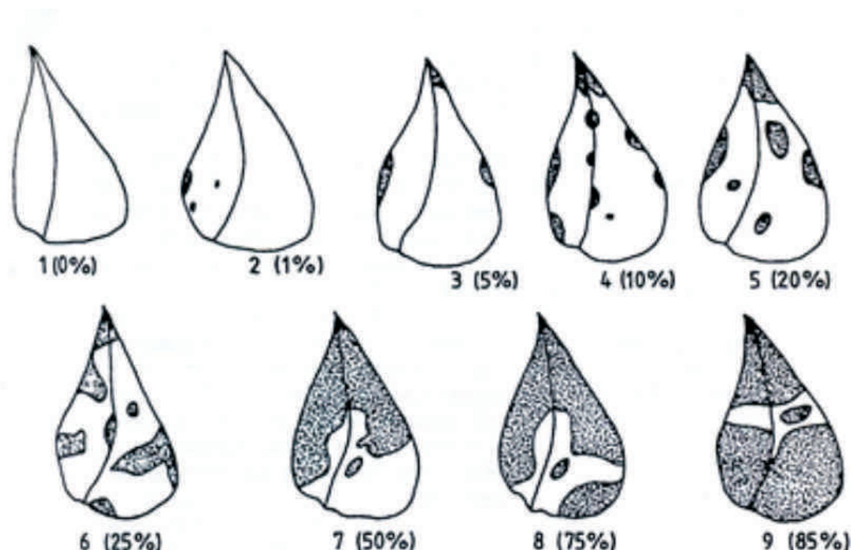

Figure 1: showing the scale used to rate common bacterial blight of beans on leaves (Opio *et al.,* 1993)

Data analysis

The data collected were analyzed statistically using analysis of variance ANOVA and means were separated using New Duncan's Multiple Range Test (NDMRT).

Results

The isolation carried on all the seed lots collected from nine cowpea producing states and three cowpea companies yielded high to moderately low bacterial population (Table 1). The highest population was obtained on seed lots collected from Kogi and Niger States, followed by Nasarawa and Kaduna states; all, in Southern Guinea savannah of the country. The lowest populations were obtained from seed lots collected from seed companies which were not statistically different from other seed lots collected from the far northern states such as Borno, Gombe, Adamawa and Sokoto (Sudan savannah of the country). Similar results were also noted for the incidence of pathogens on the seed samples.

Table 1: Incidence and population of *Xanthomonas axonopodis* pv. *vignicola* on seed samples

Isolate/Strain	Incidence %	Population
Borno (Bama)	1.0e	$2.0x10^3$e
Gombe (Akor)	1.3ed	$2.0x10^3$d
Adamawa (Mubi)	1.3ed	$2.0x10^2$d
Sokoto (Duchin)	1.0e	$2.0x10^2$e
Kaduna (Saminaka)	1.9c	$2.8x10^3$b
Kebbi (Jega)	1.4d	$1.6x10^3$c
Kogi (Idah)	3.3a	$4.1x10^3$a
Nassarawa (Keffi)	2.7b	$2.8x10^3$b
Niger (Mokwa)	3.1a	$4.1x10^3$a
Alheri	1.0e	$2.0x10^2$e
Masalaha	1.0e	$2.2x10^2$e
Premier	1.0e	$2.1x10^2$e

Figures with the same letter within a column are not significantly different at $p \leq 0.05$ using NDMRT

Biochemical/morphological tests

Twelve isolates were identified as *Xav.* All isolates produced colonies of mucoid texture on YDCA (Table 2). All the isolates were gram negative (-), produced copious amount of Extra Cellular Polysaccharide (EPS), and hydrolyzed starch, but not all developed typical yellow pigmentation on YDCA. Isolates from Kogi, Nassarawa, Niger and Kaduna produced typical yellow pigmentation on YDCA while those from Borno, Adamawa, Gombe, Sokoto, Kebbi and all the seed companies' isolates showed light yellow pigmentation. In addition, variations occurred in the number of days various colonies took to develop yellow pigment. It took three days for isolates of Kogi, Nassarawa, Adamawa and Gombe to develop typical yellow, while it took seven days for isolates of Alheri to develop creamy yellow, and Premier and Masalaha to develop light yellow pigmentation.

Pathogenicity test

The result of the pathogenicity test showed that all the isolates of *Xav* were pathogenic on the three cowpea varieties (Table 3). There was significant differences in disease incidence among the different isolates. Incidence of CoBB produced by Kogi state isolate on Sampea7 was the highest (94.00 %) but was statistically comparable to other isolates. Similar trend was observed from other isolates on Ife brown and IT86D-721. Severity of CoBB on Sampea 7 induced by Kogi state isolate was highest (8.3) which was statistically comparable to Nassarawa and Niger State. This was followed by Kaduna state isolate with the least severity induced by Adamawa state isolate (5.1), which was not statistically different from the other isolates. Similar result was observed on disease severity of the isolates on IT86D-721. The typical symptoms of CoBB were produced three days

Table 2: Result of Biochemical/morphological test

Isolate/Strain	Gram reaction[1]	Pigmentation[2]	EPS[3]	Starch[4]	No. of days to pigmentation appearance[5]
Borno (Bama)	-	T	+	+	5
Gombe (Akor)	-	LY	+	+	3
Adamawa (Mubi)	-	T	+	+	3
Sokoto (Duchin)	-	CY	+	+	4
Kaduna (Saminaka)	-	T	+	+	5
Kebbi (Jega)	-	T	+	+	4
Kogi (Idah)	-	LY	+	+	3
Nassarawa (Keffi)	-	T	+	+	3
Niger (Mokwa)	-	T	+	+	4
Alheri	-	CY	+	+	7
Masalaha	-	T	+	+	7
Premier	-	T	+	+	7

[1]= - = negative reaction, + = positive reaction,
[2]= T = Typical yellow, Ly = Light Yellow, CY = Creamy yellow. [3] EPS = extra cellular polysaccharide, [4] = a clear zone, indicating starch hydrolysis on YDC, [5] = days taken to form typical yellow pigmentation.

Table 3: Pathogenicity test of the isolates on three cowpea varieties

Isolates	Sampea 7		Ife brown		IT86D-721		Days to symptom appearance
	Incidence	Severity	Incidence	Severity	Incidence	Severity	
Borno (Bama)	71.15ab	5.4de	60.50df	4.1b	61.50c	4.7d	7.0e
Gombe (Akor)	93.50ab	5.3de	60.40df	4.2b	62.40c	4.6d	7.0e
Adamawa (Mubi)	73.70ab	5.1de	65.00df	3.8bc	60.00c	4.9d	7.0e
Sokoto (Duchin)	73.10ab	5.2de	59.90df	3.7bc	61.50c	4.8d	6.0d
Kaduna (Saminaka)	82.75ab	6.8c	75.80c	5.0a	75.50b	6.0ab	6.0d
Kebbi (Jega)	79.50ab	5.4d	60.50fg	3.9bc	74.90b	4.7d	4.0b
Kogi (Idah)	94.00ab	8.3a	81.75a	5.3a	82.00a	6.8a	3.0a
Nassarawa (Keffi)	93.50ab	7.5b	81.20a	5.2a	79.30a	6.4a	3.0a
Niger (Mokwa)	90.60ab	7.2ab	78.50b	5.1a	80.00a	6.1ab	5.0c
Alheri	74.05ab	5.4d	63.20e	3.9bc	61.00c	6.0abc	4.0b
Masalaha	75.05ab	5.1de	60.40fg	4.1b	61.00c	5.2bcd	4.0b
Premier	71.85ab	5.4d	61.40f	3.8bc	62.60	5.0cd	5.0c

Figures with the same letter within a column are not significantly different at $p \leq 0.05$ using DMRT, severity assessed on a scale of 1-9

after inoculation of the isolates from Kogi and Niger; Alheri, Kebbi and Masalaha isolates took four days; Premier and Niger isolates took five days; Kaduna and Sokoto isolates took six days; while, that of Adamawa, Gombe and Masalaha isolates took seven days to develop typical symptoms. CoBB disease symptoms appeared first as water-soaked spots followed by chlorosis and necrosis on leaves. Although isolates from Kogi, Nasarawa and Niger appeared to be more virulent, all other isolates demonstrated variable level of virulence.

Discussion

The study demonstrated that the cowpea seeds used by farmers in Nigeria were infected by *Xav* both from seeds procured in local markets and seeds produced by seed companies. The lowest level of seed infection was 1.0 % and the bacterial population for seed lots infection ranged between 10^2-10^3 cfu/100 seeds. The minimum bacteria population necessary to incite infection in the field was found to be 10^2 cfu/kg of seeds (Opio *et al*., 1993). The low incidence and severity of *Xav* on seeds collected from Borno, Gombe, Adamawa, Kebbi and Sokoto and that of seed companies could be as a result of environmental conditions and effect of seed treatment respectively (Andrew, 1990). All seeds collected from seeds companies were treated with protective chemicals; Borno, Gombe, Adamawa, Kebbi and Sokoto are located in Northern Guinea savannah where the hot season is longer and more severe, these may account for the lower incidence from these seed lots. Liao and Shollenberger (2003) reported that bacterial cells that had been exposed to high temperature (physical stress) and some form of chemical treatment have difficulty growing on selective and semi-selective media like YDCA. However, the injury or stress does not affect their pathogenic ability on the host. A level of seed infection of 0.02% was found to initiate and cause severe infection in the field

(Schaad, 1988). The characteristics of all the bacteria isolates from selected locations across the cowpea producing regions shows that all identified as *Xav*. Most of them produced typical yellow pigment (Xanthomonadin) with few showing variable pigmentation. The typical yellow colour of the pigment is as a result of the presence of a particular carotenoid pigment which is generally indicative of genus *Xanthomonas* and the concentration of the carotenoid vary from strain to strain (Gottward *et al*., 2002; Masterson, 2007).

The high level of virulence of all the isolates on the three cowpea cultivars reflects the cultivation of these cultivars in all the regions and, thus, the selection pressure to evolve pathogenicity on the cultivars. This result parallels that of Alexander *et al.* (2004) who had reported that the pathogenicity of East Africa *X .campestris* pv. *phaseoli* strains on two common bean gene pools was as a result of this strain being adapted to be pathogenic on them over time as a result intensive cultivation of these cultivars. The variation observed in pathogenicity of the isolates may be due to geographical and ecological differences in their origin (Ivey *et al*., 2007), since most cowpea producing regions in Nigeria have considerable climatic differences. Geographical location and different cropping pattern have been reported to influence the pathogenicity of the pathogen (Shoaga, 1998; Ivey *et al*., 2007; Okechukwu *et al*., 2010). The incidence, population and high pathogenicity of isolates from Kogi, Niger and Nassarawa respectively reveal their high virulence. These three states share almost the same ecological and climatic conditions. High humidity, temperatures fluctuating between 20 – 25°C and alternating dry and wet weather can cause more than 40 % yield loss in susceptible cultivars (Serracin *et al.,* 1991). This is also in line with Gottward (2002) who reported that the spread and pathogenicity of *Xanthomonas axonopodis* pv. *citri* are limited by temperature. The diversity among the isolates may be responsible for the wide spread of the pathogens in virtually all the cowpea producing states in northern Nigeria. Together, these results indicate the presence of *Xav* in all the cowpea producing states and variability exist in their biochemical, morphological and pathogenic characteristics. The results have identified the type or types of CoBB bacteria prevalent in the region in order to select appropriate virulent strains to use when breeding for resistance. The identification of these pathogenic diversity in CoBB occurring in the locations is a necessary step towards breeding resistant varieties against the disease. In addition, with very few management options available, it is clear that a better understanding of the population structure of this highly diverse pathogen is needed in order to develop pathogen-targeted and, possibly, geographically targeted management practices.

Conclusion

Xanthomonas axonopodis pv. *vignicola* was present in all the seed samples collected from the seed companies and those from the local markets in cowpea producing locations in Northern Nigeria. Diversity was established among the isolates collected in terms of pathogenicity, biochemical and morphological characteristics.

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