

MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF STRAINS OF *Xanthomonas axonopodis* pv. *vignicola* ISOLATED FROM COWPEA GROWN IN THREE AGROECOLOGICAL ZONES IN NIGERIA

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ABSTRACT

Cowpea bacterial blight (*Xanthomonas axonopodis* pv *vignicola*) is a devastating disease of cowpea in Nigeria. Accurate identification is the first step for an effective management option. Thus, *X. axonopodis* pv. *vignicola* isolated from cowpea (*Vigna unguiculata* (L.) Walp) grown in different Agro-ecological zones in Nigeria were characterized using morphological characteristics, biochemical and antibiotic sensitivity tests. Pot experiments were conducted on two susceptible lines of cowpea (IT90k – 76) and (IT84s – 2246 – 4) in the screen house. In vivo infectivity studies were carried out to determine the effect of each of the *X. axonopodis* pv. *vignicola* isolate groups on the cowpea. All isolates from each of the agroecological zones were classified into Group 1 (light yellow) and Group 2 (brownish yellow) based on their morphological presentation on nutrient agar medium. Results from four of the biochemical test showed differences between the two morphologically different groups. Group 2 isolates showed a large zone of inhibition to a Lincomycin hydrochloride while isolates from Group 1 were not affected by the action of the same antibiotic. Blight symptom was only observed when the two types of isolates were combined and inoculated simultaneously. This study showed that there are two morpho-types of *X. axonopodis* pv. *vignicola* and their synergetic infectivity results in blight symptoms expression in cowpea.

Key words: Bacterial blight, Morpho-types, Isolates, synergy, antibiotics

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INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important grain legume throughout the tropics and subtropics because it does well where other food legumes fail to perform. As food, cowpea is eaten in the form of grain, green pods, and leaves. The leaves also serve as forage (Ball *et al.*, 2007) and a herb in most parts of Africa (Adejumo, 1997). The roots are eaten in Sudan and Ethiopia, and the peduncles and stems are used as fibers in Nigeria (Adejumo, 1997). Cowpea seeds contain 24% protein, 62% soluble carbohydrates, and small amounts of

other nutrients (Jayathilak *et al.*, 2018). Cowpea is cultivated on at least 12.57 million hectares in the world with an annual production of over 5.8million metric tons (FAO, 2019). Africa produces 95% of the world total and more than 66.75% of the world cowpea comes from the drier regions of Nigeria, Cameroon, Niger and Burkina Faso (FAO, 2019). Despite all the good agronomic attributes of this crop, the production constraints have been insects and diseases damage which often infest /infect the plant throughout its life cycle and storage (Singh *et sl.*, 1990; Emechebe and Shoyinka, 1985; Vauterin *et al.*, 1995;

Emechebe and Lagoke, 2002; Okechukwu and Ekpo, 2008; Kamara *et al.*, 2013). Other constraints are drought, excessive moisture, temperature extremes, late maturity, and poor plant types (Muleba and Ezumah, 1985).

Bacterial diseases reported on cowpea are bacterial blight (*Xanthomonas axonopodis* pv. *vignicola*), bacterial pustule (*X. axonopodis* pv. *vignae*), bacterial wilt (*Pseudomonas syringae* pv. *solanacearum*) and halo blight (*P. syringae* pv. *tabaci*) (Emechebe and Shoyinka, 1985; Emechebe and Florini, 1997; Okechukwu and Ekpo, 2008). Bacterial blight is one of the most destructive diseases in cowpea producing areas with widespread distribution (Patel, 1985; Vauterin *et al.*, 1995; Okechukwu and Ekpo, 2008; Claudius-Cole *et al.*, 2014) with bacterial pustule having limited distribution (Emechebe and Florini, 1997; Emechebe and Lagoke, 2002; Okechukwu and Ekpo, 2008; Claudius-Cole *et al.*, 2014). Yield loss due to *X. campestris* pv. *vignicola* ranges between 3-100% depending the strain, cultivars and stage of growth at the time of infection (Shoaga *et al.*, 2001; Okechukwu and Ekpo 2008; Okechukwu *et al.*, 2010). Among different strategies to control cowpea bacterial blight, the development of resistant cultivars would be most attractive to farmers (Emechebe and Shoyinka 1985; Khatri-Chhetri *et al.*, 2003; Agbicodo *et al.*, 2010) in Africa. Several reports (Okechukwu and Ekpo, 2004; Agbicodo *et al.*, 2010; Duche *et al.*, 2015) on resistance to *X. campestris* pv. *vignicola* exist but this largely depends on the isolates of the bacterium (Shoaga *et al.*, 2001; Agbicodo *et al.*, 2010; Okechukwu *et al.*, 2010; Duche *et al.*, 2015).

Identification of *X. campestris* pv. *vignicola* isolates from different agro-ecologies in Nigeria was mainly based only on virulence (Shoaga *et al.*, 2001; Okechukwu and Ekpo, 2004; Agbicodo *et al.*, 2010; Ganiyu *et al.*, 2017; Durojaye *et al.*, 2019) which could lead to non-repeatability of the experiment. Identification/characterization of a pathogen is a key for assessment of plant resistance,

ensure repeatability and reduce variability of experimental results. Information on the identity of strain peculiar to an agro-ecological zone is critical to efforts aimed at screening cowpea cultivars for resistance as this status is isolates/strains dependent. For example IT81D-1228-14 was reported resistant (Agbicodo *et al.*, 2010) and susceptible (Durojaye *et al.*, 2019), mainly due to use of different non-characterized isolates (Duche *et al.*, 2015). Thus, the aim of this study was to characterize and determine the pathogenicity of different isolates of *X. campestris* pv. *vignicola* from three agro-ecological zones in Nigeria.

MATERIALS AND METHOD

Sample collection site

States in three agro-ecological zones of Guinea savanna (Kano, Kaduna, Niger), humid forest (Cross River, Ebonyi, Edo state) and rain forest (Oyo, Ogun, Ondo) in Nigeria were visited (Fig. 1). Three Local Government Areas (LGA) per state were visited with bacterial blight symptomatic leaves collected randomly on 1-2 month cowpea plants from three farmers' fields per LGA (243 fields in total). Leaves were wrapped in used newspapers and labelled with the farm sites, geographical coordinate, and date of sampling and placed in a box.

Isolation of bacterial blight pathogen

Isolation of *X. axonopodis* pv. *vignicola* was carried out on each of the samples by excise about 2mm of the infected site on the leaves and tease them in few drops of sterile distilled water and streaked on Nutrient agar (NA). This was incubated at 27°C for 48hr and was purified thereafter to obtain pure culture. Culture of *X. axonopodis* pv. *vignicola* was preserved on NA slant at 4°C until needed.

MORPHOLOGICAL AND BIOCHEMICAL TESTS

Morphological characteristics

Growth characteristics on Nutrient Agar, Gram test and Microscopic examination were used to determine the morphological characteristics. Isolates were streaked on

nutrient agar and incubated at 28°C for 48 hours. Growth color was recorded.

Gram reaction

Gram test of the isolates followed the Gram staining procedure of Schaad, (2001) Thinly spread bacterial smear was prepared on a clean slide, air-dried and fixed by passing thrice over a spirit lamp flame (. The smear was flooded with crystal violet solution (1 minute) and rinsed with distilled water for few seconds and excess water drained off and blot dried. It was followed by flooding with iodine solution (1 minute) and rinsed again with distilled water for few seconds and blot dried. The slide was flooded with decolorizer using 95% ethanol until the solvent flow colorlessly from the slide and rinsed again with distilled water (2 seconds). The slide was counter stained for about 10s with Safranin, washed in distilled water, blot dried and examined under microscope using oil immersion objective (x100).

Biochemical tests

Five biochemical tests (Esculin hydrolysis, Oxidation/Fermentation, Starch hydrolysis, Milk proteolysis and Gelatin hydrolysis) and seven antibiotics were used to characterize the isolates and determine their reactions to different antibiotics respectively.

Esculin Hydrolysis

Esculin broth consists in g/l of yeast extract, 5g; NaCl, 5g; MgSO₄.7H₂O, 0.2g K₂HPO₄, 0.5g; NH₄H₂PO₄, 0.5g; ferric ammonium citrate 50mg, and esculin (sigma) 1g, was prepared and adjusted to pH 6.8. About 7mls of the mixture was dispensed in glass vials and sterilized. A loopful of a 48hr bacterial culture was inoculated in each tube with the control left uninoculated on a rotating shaker at 28°C. The reaction started after the 5th day and readings was taken on the 10th day. Complete Hydrolysis results in lack of UV light fluorescence of esculin

Oxidation/Fermentation Test

Eschenlauer *et al.*, (2002) and Yendrembam *et al.*, (2020) described a method for fermentation assays that avoid masking of

acid production by ammonia production. The medium contains (g/l) peptone, 2.0; NaCl, 5.0; KH₂PO₄, 0.3; agar (Difco), 3.0 and 3ml/l of a 1% aqueous solution of bromothymol blue. The compounds were dissolved and pH was adjusted to 7.1. The medium was dispensed 5ml aliquots into 13mm diameter test tubes and sterilized at 121°C for 10- 15 min. A 10% glucose solution was filter sterilized and 0.5ml is aseptically added to each tube. Two tubes were stab inoculated for each of the isolate from each location. One was sealed with sterile liquid paraffin to a depth of 2 cm and the other was not. A color change in the anaerobic tube demonstrates glucose fermentation i.e. fermentation ability. Positive and negative controls consist of *Erwinia* and *Pseudomonas* isolates respectively.

Starch Hydrolysis

Nutrient agar plates containing 0.2% soluble starch (w/v) was prepared and inoculated with the different isolates and incubated at 28°C until growths occurred. Plates were then flooded with iodine solution (iodine 1.0g, potassium iodide 2.0g, distilled water 100ml). A clear zone around a colony is a positive reaction for starch hydrolysis while negative reaction gives the blue-black stain of unutilized starch.

Milk Proteolysis

Reconstituted powdered milk with 40 mg/l of bromocresol purple was steamed sterilized for 30min, and these was done daily for three consecutive days, then the mixture was aseptically dispensed into sterile tubes and inoculated with the isolates using a loopful of cells and this was incubated at 27°C, for 3 days. Casein (milk protein) digestion is indicated by clearing reaction after incubation.

Gelatin hydrolysis

Nutrient agar with 0.4% of gelatin was poured into Petri dishes, cooled and dried overnight. The plates were inoculated with the isolates and incubated at 27°C for 3 days. The plate surface was then flooded with 5 ml mercuric chloride (Toxic) solution

(HgCl₂, 12g; distilled water, 80mls; concentrated HCl, 16ml). A clear zone surrounding bacterial growth indicates positive reaction for gelatin hydrolysis (Tille and Forbes, 2014).

Antibiotics test

Gentamycin (50ug/ml dissolved in water), Chloramphenicol (20ug/ml dissolved in Absolute ethanol), Streptomycin sulphate (50ug/ml dissolved in water), Lincomycin hydrochloride (10ug/ml dissolved in water), Vancomycin (50ug/ml dissolved in water), Phosphomycin (50ug/ml dissolved in water) and Cefazolin (10ug/ml dissolved in water) were used to determine the isolates reaction to different antibiotics.

Paper discs of about 6 mm diameter used in the experiments were improvised in the laboratory by cutting glass micro fibre filters into the required size needed for this work. Bacterial strains evaluated for sensitivity to antibiotics were prepared by suspending a loopful of 48hr – old bacterial culture in 20ml of sterile distilled water (SDW). The resulting bacterial suspension was then adjusted to an optical density of 0.06 at 620 nm (which corresponded to ca. 10⁸cfu /ml). One micro liter of the bacterial suspension was spread on Nutrient agar surface in Petri dishes using a Drigalski spreader. Then, paper discs were soaked in the antibiotic's solution, laid on the agar surface and labelled accordingly. Inoculated plates were incubated at 28°C for 48 h and observed daily for growth inhibition zones around the paper discs. Radial inhibition zones around each disc, corresponding to the level of antibiotic activity for each treatment were measured using ruler.

Pathogenicity test

Pathogenicity test were conducted on two susceptible lines of cowpea (IT90k-76) and (IT84s-2246-4) in the screen house of Germplasm Health Unit in IITA in order to determine the infective capacity of *X. axonopodis* pv *vignicola* isolates on the cowpea lines. Bacterial colonies from 24hr nutrient agar culture plates were washed off with sterile normal saline (0.85g of NaCl in

1 liter of SDW) into a conical flask. An inoculum concentration of 10⁶ colony forming unit (CFU)/ml was prepared from this stock by using a Milton Roy spectrophotometer (Spectronic 21 model) set at a wavelength of 640nm. A bacteria concentration of 10⁷ CFU/ml is obtained when the optical density reading is 0.009 absorbance units (Myers *et al.*, 2013).

The prepared inoculum was stored in a 600ml conical flask in the refrigerator immediately after preparation. It was later transferred on ice prior to inoculation to inhibit proliferation of bacterial cells after counting. Inoculation was done within 35min of calibration of the inoculum. The two isolates were inoculated individually on 2 weeks old plants from the two varieties in one set of the experiment while the two isolates were combine at 1:1 ratio to inoculates plants from the two accessions. The plants were covered with transparent polythene sheet to provide humidity for initiation of infection within 48hr after which this was removed and presence or absence of characteristic blight symptoms was recorded for up to 28 days.

RESULT

All isolates irrespective of agro-ecological zones have similar morphology and biochemical characteristics, thus Ebonyi, Mokwa and Ikenne isolates were selected to represent humid forest, Guinea savanna and rain forest respectively in the figures and tables. Isolates from each of the agro-ecological zones were Gram negative, mucoid, convex and had two colour presentations. These presentations were classified into two isolates: Isolate 1 light yellow and Isolate 2 brownish yellow (Fig. 2). Biochemical test conducted on the two isolates from each agro-ecological zone show that, the two isolates were negative to oxidation/fermentation test (Table 1). Isolate 1 (light yellow) from all the zones was negative to esculin, gelatin, starch and Milk proteolysis while all isolate 2 (brownish yellow) from each zones were positive to all the tests (Table 1).

Three out of the seven antibiotics used differentiated the isolates obtained from each agro-ecological zones (Table 2). Lincomycin hydrochloride restricted the growth of isolate 2 to form inhibitory zones around the antibiotics disc used whereas isolate 1 of each zone were able to grow on the same set of antibiotics disc (Fig. 3). On the contrary Isolate 1 formed inhibitory zones around the antibiotics disc where Vancomycin and Cefazolin was used, that is, the two antibiotics restricted the growth of the bacteria around where the discs containing the antibiotics were placed. Gentamycin, Chloramphenicol, Streptomycin sulphate and Phosphomycin did not inhibit any of the isolates, as all of them thrived on these antibiotics.

The screen house infectivity tests conducted on the two susceptible lines of cowpea showed that, each of the bacteria isolate types could not produce blight symptoms individually. Blight symptoms were obtained on the plants only when inoculation was done using a mixture of the two bacteria isolates types (Fig. 4).

DISCUSSION AND CONCLUSION

The study was conducted to characterize different strains/isolates of *Xanthomonas axonopodis* pv *vignicola* from different locations in nine (9) states representing three agro-ecological zones of Nigeria. It was hypothesized that resistance developed against a strain in a zone will not be effective on another strain in a different zone without appropriate information on the characteristics needed for identification of the isolates. Across the different zones visited, two different isolates of *X. axonopodis* pv *vignicola* were distinguishable from same infected plant samples collected, This was similar to the observation of Verdier *et al.* (1998) of two strains isolated from the same plant with different colony types but with same type of symptom. All the isolates obtained from the study irrespective of location showed phenotypic characteristics of *Xanthomonas axonopodis* pv. *vignicola* being Gram-

negative, rod shaped with yellow, convex colonies (Nandini, 2012; Hayatu *et al.*, 2013; Duche *et al.*, 2015). These two isolates were tagged 1 and 2 based on their phenotypic expressions on culture medium, biochemical and antibiotic reagents suggesting the existence of strains/morphotypes of *X. axonopodis* pv *vignicola* (Fourie *et al.*, 2011; Nandini, 2012). Isolates 1 had similar phenotypic characteristics irrespective of location ditto for isolates 2, which confirms the report of Duche *et al.*, 2015 of high levels of similarity within the same zone. The similar characteristics among the zones reveal seedborne patterns of pathogen migration. Farmers use and exchange their previous season's farm saved seeds which have been recycled over many years with build-up of seedborne pathogen which supported the reports of Claudius-Cole *et al.* (2014) and Ganiyu *et al.* (2017) that seeds contribute the largest percentage to the spread of *X. axonopodis* pv. *vignicola*). It also indicates that the colony colour can be used as an indicator of differences in strains/morphotypes.

On culture medium, isolate 1 was observed to be light yellow which is similar to the report of Nandini (2012) while isolate 2 was brownish yellow in this study but described by Nandini (2012) as pale/dark yellow. Duche *et al.* (2015) also reported variation in colour ranging from yellow to yellow creamy colony colour on nutrient agar medium with 5% glucose which was attributed to Xanthomonadin pigment production. Nevertheless, this also goes to show the need for characterization of the isolates before they can be used for further studies.

Studies on the two isolates (1 and 2) when subjected to esculin, gelatin, milk proteolysis and starch hydrolysis tests described by Aidan *et al.* (2012) shows that isolate 2 in each case completely hydrolyzed the medium while isolate 1 could not hydrolyze any of the media. Duche *et al.* (2015) reported different degree of variation in the

isolates to oxidase reaction, casein hydrolysis and starch hydrolysis which was attributed to variations among isolates. The biochemical reaction coupled with the pale yellow colony colour of isolate from Nandini (2012) gives an indication that it might be similar to isolate 2 from this study. This suggests that the colony colour and biochemical reactions can be used also to identify/characterize this pathogen.

Bioefficacy of different antibiotics used in this study indicated that Isolate 2 was inhibited by Lincomycin hydrochloride with no effect on isolate 1. When vancomycin and cefazolin were used, the effect was only on isolate 1. This observation supported the works by Nandini (2012) of differences in the isolate reaction based on the antibiotics used indicating that the antibiotic reactions can also be used to rate the pathogens.

Pathogenic variation of isolates used in previous studies for assessments of resistance was mainly attributed to the symptom expression from the isolates (Duche *et al.*, 2015; Durojaiye *et al.*, 2019). Different strains within *X. campestris* pv. *vignicola* have been reported responsible for this variation based on the level of virulence on cowpea cultivars (Agbicodo, *et al.*, 2010). This was described by Khatri-Chhetri *et al.* (2003) as low, medium and high virulence. In this study, virulence was obtained only when the two isolates were combined and co-inoculated. This was similar with Fourie *et al.* (2011) report on the use of two mixture of isolates (X6 and Xf105) for inoculation to improve symptom expression. This suggests that symptoms expression of a typical cowpea bacterial blight would only occur when the isolates from each of the two strains are present. This has been reported (Kaur *et al.*, 2011; Lamichhane and Venturi, 2015) as pathogen-pathogen interactions or synergy

which improves the ability of the pathogens to cause infection in combination than individually. The mechanism(s) for this synergy among different bacterial species is currently unknown (Lamichhane and Venturi, 2015).

In this study, we found that purification of colonies to differentiate the two isolates is important to prevent unintended synergy between the isolates which will give false information on the virulence of the isolates. We also agree with Verdier *et al.* (1998) and Khatri-Chhetri *et al.* (2003) that strains of *X. axonopodis* pv. *vignicola*, as well as those found from this study acting synergistically, belonged to the same pathovar based on our findings on pathological, biochemical and antibiotic tests. We support the view of Verdier *et al.* (1998) and Duche *et al.* (2015) of the non-existence of races or distinct pathovars of *X. axonopodis* pv. *vignicola*. In our view, this pathogenic variation are results of synergism among the different strains, however this hypothesis needs further molecular clarification as done for strains of *X. oryzae* pv. *oryzae* (Vera Cruz *et al.*, 1996). Thus, these synergistic interactions is important for the understanding of pathogenesis of the strains in cowpea for the development of effective disease control strategies as suggested by Lamichhane and Venturi (2015).

It is therefore evident from the study that the two morphotypes isolates are different and react in synergy to produce symptoms of bacterial blight which is contrary to a lot of research earlier done on this pathogen. Further research, which could not be undertaken due to paucity of funds, needs to focus on molecular characterization of the strains from different zones and molecular mechanism of host-pathogen interactions to enhance understanding and promote efforts in managements of the disease.

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APPENDIX

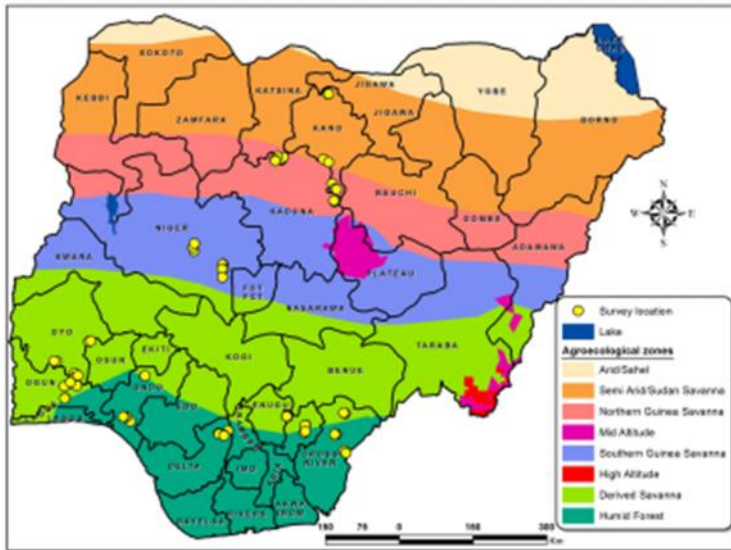


Fig. 1 Map showing collection sites in different agro-ecological zones in Nigeria

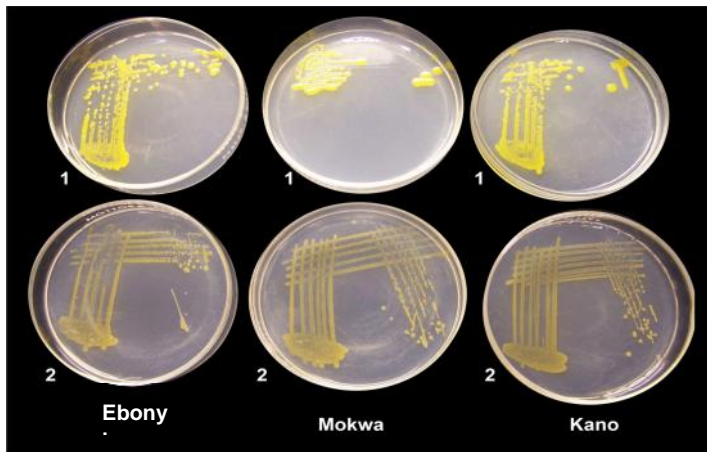


Fig. 2 Isolates of *Xanthomonas axonopodis* pv *vignicola* from different agroecological zones with colour presentations of (1) light yellow (2) brownish yellow

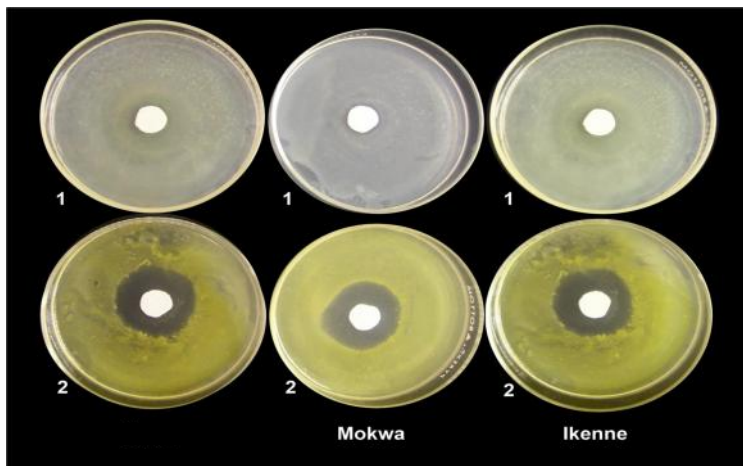


Fig.3 Growth of isolate on *Lincomycin* hydrochloride

Table 1: Biochemical reactions of the Isolates from each of the agro-ecological zones

Samples	Oxidation/ Fermentation	Esculin Hydrolysis	Starch Hydrolysis	Milk Proteolysis	Gelatine Hydrolysis
Ikenne					
Isolate 1	- ve	- ve	- ve	- ve	- ve
Isolate 2	- ve	+ ve	+ ve	+ ve	+ ve
Mokwa					
Isolate 1	- ve	- ve	- ve	- ve	- ve
Isolate 2	- ve	+ ve	+ ve	+ ve	+ ve
Ebonyi					
Isolate 1	- ve	- ve	- ve	- ve	- ve
Isolate 2	- ve	+ ve	+ ve	+ ve	+ ve

Table 2: Effect of antibiotics on the two isolates of *Xanthomonas axonopodis pv vignicola*

Antibiotics	Isolate 1	Isolate 2
Gentamycin	-	-
Chloramphenicol	-	-
Streptomycin sulphate	-	-
Lincomycin hydrochloride	-	+
Vancomycin	+	-
Phosphomycin	-	-
Cefazolin	+	-

+ = Presence of clear zone of inhibition – = Absence of zone of inhibition.

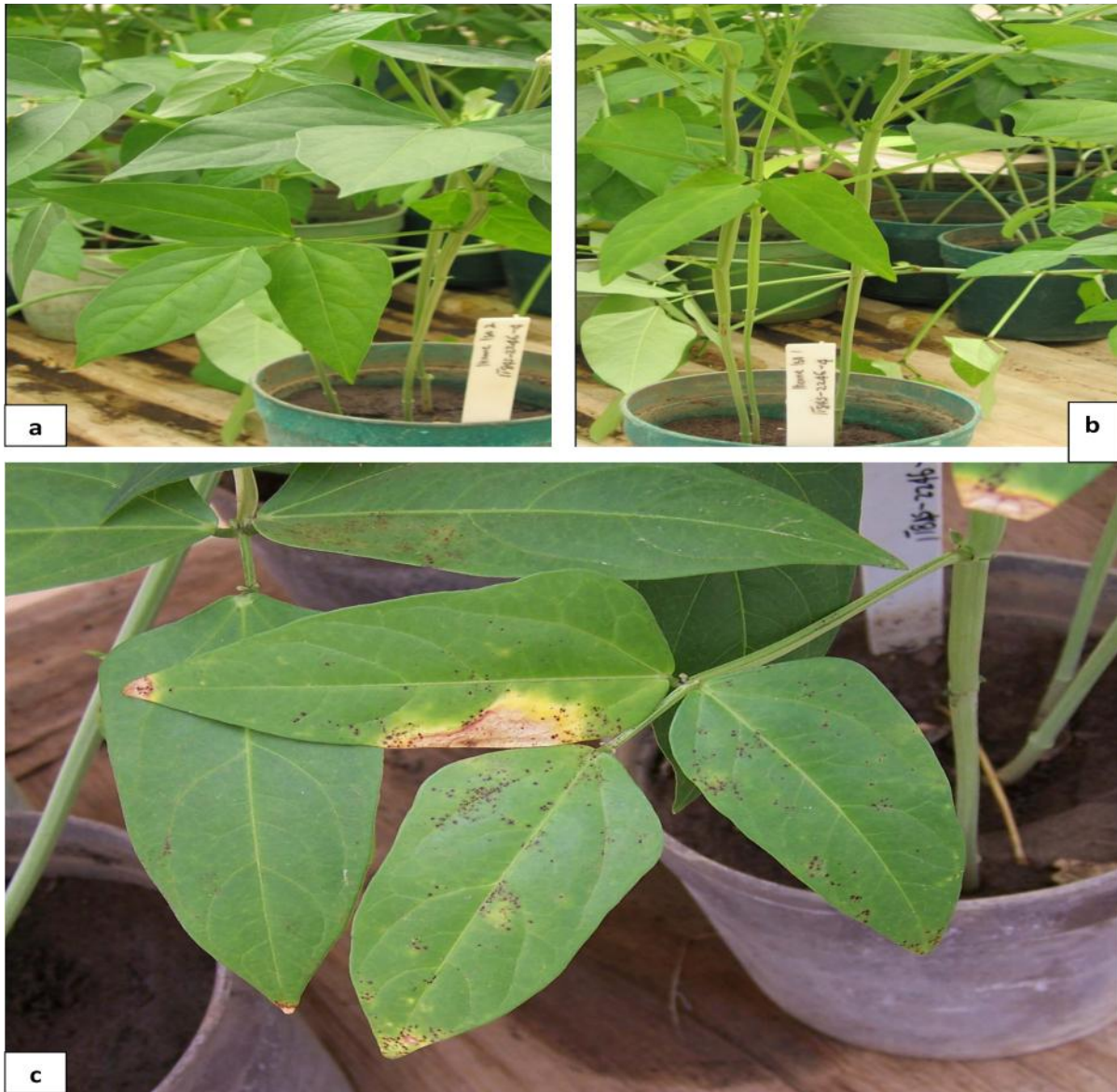


Figure 3: Cowpea plants (a) and (b) without blight symptom after 15 days of inoculation with isolate 1 and isolate 2 bacteria respectively and (c) with blight symptoms after 15 days inoculation with combined isolates of 1 & 2 bacteria.